From the Department of Medicine, Solna
Karolinska Institutet, Stockholm, Sweden

Role and therapeutic potential of non-coding RNAs in vascular remodeling and atherosclerotic plaque formation

Suzanne M. Eken

Karolinska Institutet

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Role and therapeutic potential of non-coding RNAs in vascular remodeling and atherosclerotic plaque formation

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By

Suzanne M. Eken

Principal Supervisor:
Lars Maegdefessel
Karolinska Institutet
Department of Medicine
Division of Molecular Vascular Medicine

Co-supervisors:
Per Eriksson
Karolinska Institutet
Department of Medicine
Division of Cardiovascular Genetics and Genomics

Cecilia Österholm Corbascio
Karolinska Institutet
Department of Molecular Medicine and Surgery
Division of Cardiothoracic Surgery

Opponent:
Dr. Alan Daugherty
University of Kentucky
Department of Physiology and Saha
Cardiovascular Research Center

Examination Board:
Dr. Karl Swärd
Lund University
Department of Experimental Medical Science
Division of Cellular Biomechanics

Dr. Andor Pivarcsi
Karolinska Institutet
Department of Medicine
Division of Experimental Dermatology

Dr. Maja Jagodic
Karolinska Institutet
Department of Clinical Neuroscience
Division of Epigenetic Origins and Mechanisms in Neuroinflammation

"If written in the three-letter words of the four-letter alphabet, a human being is determined by a genetic narrative long enough to fill the equivalent of 500 Bibles. In the meantime human beings have discovered this for themselves. That's right. They have uncovered our profoundest concept – namely, that life is ultimately reading. They themselves are the Book of Books."

– Harry Mulisch, *The Discovery of Heaven*, 1992

ABSTRACT

Atherosclerosis and its clinical sequelae remain a world leading cause of disease and death, despite recent advances in primary and secondary prevention. The silently progressive character of the disease, in combination with the influence of individual patient characteristics, makes acute events difficult to predict and prevent. There is a need for (1) noninvasive, accurate diagnostic methods and (2) individually tailored therapies, in order to provide effective treatment whilst avoiding unnecessary interventions and iatrogenic damage. In the search for novel detection methods and drugs, non-coding RNA has emerged as a class of important biological regulators, being crucially involved in virtually every cellular process. We show that patients at risk for cardiovascular events display characteristic non-coding RNA patterns and could be treated with RNA interference (RNAi) therapy, targeting and normalizing previously 'undruggable' physiological disruptions.

In order to measure up to these high expectations, findings from basic non-coding RNA research need to be applied in (pre-)clinical studies. MicroRNAs (miRNA, miR) have been demonstrated to be modifiers of cardiovascular disease via posttranscriptional inhibition of messenger RNA. In this thesis, *in vitro* as well as *in vivo* modulation of clinically relevant miRNAs are presented as a therapeutic approach to alter vascular cell behavior and induce reparative arterial remodeling in three cardiovascular diseases (stroke, abdominal aortic aneurysm (AAA) and radiation-induced vasculopathy). In two different mouse models of AAA, we showed that miR-24 treatment hampers AAA expansion. Cell and human tissue experiments demonstrated a positive effect of miR-24 that could be attributed to an inhibitory action of this miRNA on *chitinase 3-like 1*, a marker of macrophage-induced inflammation. In AAA, a disease for which screening has been suggested but fails to meet socioeconomic demands, we are the first to present a sufficiently powered biomarker study, where miR-99b, detected in the circulation, could predict aneurysm expansion and rupture risk. In patients with carotid artery atherosclerosis at risk for stroke, miR-210 was decreased in the atherosclerotic fibrous cap. *In vivo* experiments of murine carotid injury and plaque rupture showed that low expression of miR-210 was associated with fibrous cap smooth muscle cell apoptosis through *adenomatous polyposis coli*, and that miR-210 treatment could prevent carotid plaque rupture in mice. In chronic arterial inflammation secondary to radiotherapy, miR-29b deregulation demonstrated an adverse inflammatory and fibrotic response, which in mice could be corrected with miR-29b mimic therapy, partly through a restored inhibition of miR-29b targets *pentraxin 3* and *dipeptidyl-peptidase 4*.

Our approaches have not only revealed possible diagnostic and therapeutic use of non-coding RNAs, but have also presented us with the difficulties and limitations of presenting exogenous RNA modifiers to a diseased circulatory system. Distribution, efficacy, off-target effects, and pleiotropy are issues that need to be addressed before non-coding RNAi therapy can be exploited clinically.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärt- och kärlsjukdomar fortsätter att vara en världsledande orsak till sjukdom och mortalitet, trots en ständig förbättring av behandling såväl som prevention. Det kvarstår därför ett stort behov av (1) metoder för at fastställa diagnos på ett tidigt stadium, och (2) individuell terapi. Som ett led i att utforska nya diagnostiska metoder samt behandling, har icke-kodande RNA nyligen upptäckts som en klass av viktiga biologiska regulatorer vilka har en viktig funktion i nästan alla mänskliga cellulära processer. I denna avhandling visas att patienter som har hög risk för att utveckla hjärt- och kärlsjukdomar, uppvisar särskilda förändringar i sitt icke-kodande RNA. Dessa förändringar skulle potentiellt kunna korrigeras genom så kallad RNA-interferens-terapi (RNAi) och därigenom dämpa sjukdomsutvecklingen i hjärt- och kärlsystemet via vägar som inte kan nås med traditionell behandling.

MicroRNA (miRNA, miR) utgör en klass av icke-kodande RNA som har visats besitta potentialen att förbättra men även försämra hjärt- och kärlsjukdomar. Detta gör de genom att reglera mängden budbärar- (messenger) RNA som i sin tur förmedlar aktivitet av gener. För tre olika sjukdomar (hjärninfarkt, bukaortaaneurysm (AAA) samt strålningsinducerad vaskulit) visar vi med hjälp av odlade celler och djurstudier, att kliniskt relevanta miRNAs kan påverkas utifrån för att influera cellers beteende och initiera reparation av kärlväggen. I två olika musmodeller av AAA demonstrerade vi att miR-24 hindrar utvidgning av aneurysm. Vidare experiment i celler och human vävnad visade att den positiva effekten av miR-24 delvis kan tillskrivas en minskning av *chitinase 3-like 1*, ett protein som vidhåller kärlväggsinflammationen. För AAA, en allvarlig sjukdom för vilken befintliga screeningmetoder i nuläget inte är genomförbara utifrån ett socioekonomiskt perspektiv, visade vi att uppmätta nivåer av miR-99b i blodet kunde bidra till att förutspå utvidgning och ruptur. I patienter med åderförkalkning i halspulsådern och en förhöjd risk för stroke, observerade vi en minskad mängd miR-210 i glattmuskelceller i åderförkalkningsplackens skyddande kapsel. Musexperiment visade att miR-210-terapi kan blockera uttrycket av proteinet *adenomatous polyposis coli*, vilket förebygger glattmuskelcellernas död och därigenom stabiliserar kapseln. I kroniskt inflammerad vävnad från uttagen pulsåder som tidigare utsatts för joniserande strålning, såg vi en minskning av miR-29b. miR-29b är viktig för att förhindra inflammation och förebygga ärrbildning. I möss kunde vi korrigera bristen av miR-29b vilket därigenom ledde till minskning av inflammationsproteinerna *pentraxin 3* och *dipeptidyl-peptidase 4*.

Vårt arbete visar att särskilda icke-kodande RNA kan bidra både till en potentiellt förbättrad diagnostik och behandling. Dock har våra studier också gjort os varse om begränsningar som denna vetenskap medför, teoretisk och praktiskt. Innan det blir möjligt att överföra RNAi-terapi till kliniken måste medicinernas effektivitet och möjliga biverkningar utforskas vidare.

SAMENVATTING

Ondanks dat atherosclerose (vaatverkalking) steeds beter voorkomen en behandeld kan worden, blijven atherosclerotische aandoeningen, in de vorm van hart- en vaatziekten, 's werelds belangrijkste bron van ziekte en sterfte. Er is behoefte aan (1) methoden om de ziekte vast te stellen zonder de patiënt schade te berokkenen, en (2) op het individu toegespitste therapieën. In de zoektocht naar nieuwe detectiemethoden en medicatie is recent niet-coderend RNA ontdekt als een groep belangrijke biologische regulatoren, die van cruciaal belang is in zo goed als alle menselijke cellulaire processen. Wij toonden aan dat patiënten met een risico op hart- en vaatziekten een karakteristiek patroon van veranderingen in het niet-coderend RNA vertonen, en dat zij behandeld kunnen worden met zogenoemde RNA-interferentie (RNAi)-therapie, die de ziekmakende verstoringen van het hart- en vaatsysteem aanpakt via wegen die met traditionele medicijnen niet bereikt kunnen worden.

MicroRNAs (miRNA, miR) vormen een klasse van niet-coderend RNA die sinds enkele decennia steeds beter bekend is komen te staan als een groep moleculen die hart- en vaatziekten kan beïnvloeden door het boodschapper- (messenger) RNA, dat signalen vanuit de celkern naar de cel doorgeeft, direct tegen te werken of te vernietigen. Voor drie ziekten (herseninfarct, aneurysma van de abdominale aorta (AAA) en stralingsgeïnduceerde vasculitis (vRTx)) laten we op cellulair niveau en in dieronderzoek zien dat bepaalde klinisch relevante miRNAs van buitenaf gereguleerd kunnen worden om zo het gedrag van cellen te kunnen beïnvloeden en herstel van de vaatwand te bevorderen. In twee verschillende muismodellen van AAA demonstreren we dat miR-24 het uitdijen van een aneurysma remt. Experimenten in cellen en op humaan weefsel lieten zien dat het positieve effect van miR-24 toe te schrijven was aan remming door miR-24 van *chitinase 3-like 1*, een eiwit dat de ontstekingsreactie in de vaatwand in stand houdt. Wij presenteren tevens de eerste studie waaraan voldoende AAA-patiënten meededen om voor grote groepen patiënten aan te tonen dat de hoeveelheid miR-99b, gemeten in het bloed, kan bijdragen aan het voorspellen van uitdijing en barsten van een AAA. In patiënten met atherosclerose van de halsslagader en een hoog risico op herseninfarct zagen we verminderde expressie van miR-210 in de gladde spiercellen van de fibreuze kap van de atherosclerotische plaque. Muisexperimenten toonden aan dat miR-210-therapie de fibreuze kap kan stabiliseren, door celdood van gladde-spiercellen te voorkomen middels remming van het eiwit *adenomatous polyposis coli*. In chronisch ontstoken weefsel van slagaders blootgesteld aan radiotherapie zagen we dat miR-29b verminderd was. miR-29b is belangrijk voor het remmen van ontsteking en het voorkomen van de vorming van littekenweefsel. In muizen kon het gebrek aan miR-29b worden gecorrigeerd, hetgeen leidde tot remming van de ontstekingseiwitten *pentraxin 3* en *dipeptidyl-peptidase 4*.

Met ons werk hebben wij laten zien dat bepaalde niet-coderende RNAs kunnen bijdragen aan diagnostiek en behandeling, maar ook dat het blootstellen van een ziek hart- en vaatstelsel aan RNAi-therapie moeilijkheden en beperkingen met zich meebrengt. Voordat deze vorm van behandeling klinisch toegepast kan worden, moeten het gedrag, de effectiviteit en de bijwerkingen van RNAi-therapie verder worden onderzocht.

LIST OF SCIENTIFIC PAPERS

I. Maegdefessel L* Spin JM*, Raaz U, Eken SM, Toh R, Azuma J, Adam M, Nakagami F, Heymann HM, Chernogubova E, Jin H, Roy J, Hultgren R, Caidahl K, Schrepfer S, Hamsten A, Eriksson P, McConnell MV, Dalman RL, Tsao PS, **miR-24 limits aortic vascular inflammation and murine abdominal aneurysm development**. *Nature Communications* 2015 Oct 31;5:5214.

II. Eken SM, Korzunowicz G, Chernogubova E, Jin H, Li Y, Roy J, Asselbergs FW, Hamsten A, Eriksson P, Raaz U, Spin JM, Tsao PS, Maegdefessel L, **MicroRNAs as novel plasma biomarkers for diagnosis and prognosis of abdominal aortic aneurysm disease**. Manuscript.

III. Eken SM, Jin H, Chernogubova E, Li Y, Simon N, Sun C, Korzunowicz G, Busch A, Bäcklund A, Österholm C, Razuvaev A, Renné T, Eckstein HH, Pelisek J, Eriksson P, Gonzalez Díez M, Perisic Matic L, Schellinger IN, Raaz U, Leeper NJ, Hansson GK, Paulsson-Berne G, Hedin U, Maegdefessel L, **MiRNA-210 Enhances Fibrous Cap Stability in Advanced Atherosclerotic Lesions**. *Circulation Research* 2017;120:633-644.

IV.- Eken SM, Christersdottir T, Sangsuwan T, Jin H, Chernogubova E, Pirault J, Sun C, Simon N, Winski G, Winter H, Tornvall P, Haghdoost S, Hansson GK, Halle M*, Maegdefessel L*, **miR-29b mediates the chronic inflammatory response in radiotherapy-induced vascular disease**. Manuscript submitted.

* contributed equally

PUBLICATIONS NOT INCLUDED IN THE THESIS

Raaz U, Zöllner AM, Schellinger IN, Toh R, Nagakami F, Brandt M, Emrich FC, Kayama Y, Eken S, Adam M, Maegdefessel L, Hertel T, Deng A, Jagger A, Buerke M, Dalman RL, Spin JM, Kuhl E, Tsao PS, **Segmental Aortic Stiffening Contributes to Experimental Aortic Aneurysm Development**, *Circulation* 2015 May 19;131(20):1783-93.

Raaz U, Schellinger IN, Chernogubova E, Warnecke C, Kayama Y, Penov K, Hennigs JK, Salomons F, Eken S, Emrich FC, Zheng WH, Adam M, Jagger A, Nagakami F, Toh R, Toyama K, Deng A, Buerke M, Maegdefessel L, Hasenfuß G, Spin JM, Tsao PS, **Transcription Factor Runx2 Promotes Aortic Fibrosis and Stiffness in Type II Diabetes Mellitus**, *Circ Res* 2015 Aug 28;117(6):513-24.

Wang D, Deuse T, Stubbendorff M, Chernogubova E, Erben RG, Eken SM, Jin H, Li Y, Busch A, Heeger CH, Behnisch B, Reichenspurner H, Robbins RC, Spin JM, Tsao PS, Schrepfer S, Maegdefessel L, **Local MicroRNA Modulation Using a Novel Anti-miR-21-Eluting Stent Effectively Prevents Experimental In-Stent Restenosis**, *Arterioscler Thromb Vasc Biol* 2015 Sep;35(9):1945-53.

SUPPORTING REVIEWS

Eken SM, Jin H, Chernogubova E, Maegdefessel L, **Making Sense in Antisense: Therapeutic Potential of Noncoding RNAs in Diabetes-Induced Vascular Dysfunction**, *J Diabetes Res* 2013,2013:834727. Review.

Busch A, Eken SM, Maegdefessel L, **Prospective and Therapeutic Screening Value of Non-Coding RNA as biomarkers in Cardiovascular Disease**, *Ann Transl Med* 2016 Jun;4(12):236. Review.

CONTENTS

LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ACTA2	Smooth muscle alpha actin
AGO2	Argonaute 2
BiKE	Biobank of Karolinska endarterectomies
BIRKA	Biobank of irradiated tissues at Karolinska
CEA	Carotid endarterectomy
circRNA	Circular ribonucleic acid
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EVAR	Endovascular aortic repair
GWAS	Genome-wide association studies
LDL	Low density lipoprotein
LNA	Locked nucleic acid
mRNA	Messenger ribonucleic acid
miRNA	Micro-ribonucleic acid
ncRNA	Non-coding ribonucleic acid
NF-κB	Nuclear factor kappa B
PEI	Polyethyleneimine
PPE	Porcine pancreatic elastase
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
RISC	Ribonucleic acid-induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNA-Seq	Ribonucleic acid sequencing
RT	Radiotherapy
SMC	Smooth muscle cell
SMART	Second manifestations of arterial disease
TCF/LEF	T-cell factor/lymphoid enhancer factor
US	Ultrasound
Wnt	Wingless-related integration site

1 INTRODUCTION

1.1 ATHEROSCLEROSIS

Atherosclerosis is defined as thickening and loss of elasticity of the inner lining of arteries. In humans, this biological process is part of normal aging[1,2]. Atherosclerosis is initiated early in life[3,4] and characterized by the development of arterial lesions commonly referred to as *plaques* or *atheromas*. Plaque development has various stages, of which most are clinically silent. Only when plaque-derived stenoses become flow-limiting, that is, in the very last stage of the disease, they manifest as cardiovascular disease[5]. Disruption of arterial blood flow means that tissue becomes deprived of oxygen and nutrients, which leads to cell death, inflammation and organ dysfunction. Examples of clinical consequences are stroke and myocardial infarction, which together account for a third of all deaths worldwide[6]. The classical view that cardiovascular disease is a problem of wealth is outdated; 80% of cardiovascular deaths occur in low- and middle-income countries[7]. Despite immense efforts from the medical and scientific communities, the worldwide hegemony of cardiovascular disease as the most important cause of death has to date not been halted.

The strongest risk factors for cardiovascular disease are age, gender, smoking, low density lipoprotein (LDL) cholesterol levels and blood pressure[8]. Some of these are modifiable with life style interventions, measures that can have an important effect on a population scale. The deployment of statins has further improved cardiovascular disease prevention: by lowering LDL cholesterol, statins are effective in reducing risk of myocardial infarction and stroke[9]. In large trials including patients with hypertension, blood pressure lowering by blocking of the renin-angiotensin-aldosterone system (RAAS) has shown to reduce cardiovascular morbidity as well as all-cause mortality[10]. Both of these successful treatments are directed towards early changes in atherosclerosis development, and for many people at high risk of cardiovascular disease, with advanced atherosclerotic plaque formation but no symptoms, these treatments may offer *too little too late*. This, as well as the availability of a rapidly growing arsenal of medication and invasive procedures (percutaneous revascularizations, endarterectomy) calls for individually tailored diagnosis and therapy. Especially in a patient population at high cardiovascular risk – the elderly – treatment risks may outweigh possible benefits[11]. Present-day 'personalized medicine' originates from these demands. Our work aims to contribute to finding new diagnostic and treatment methods that are less invasive, and more effective, in predicting and preventing cardiovascular disease, and at the same time limit an atherosclerosis carrier's risk for (further) disease manifestations while reducing overtreatment, an approach essential to harness escalating healthcare costs.

1.1.1 Pathologic basis of atherosclerosis

The least controversial and best understood risk factor for atherosclerosis is a disordered plasma lipid content (dyslipidemia), especially a high concentration of LDL cholesterol[12]. The innermost layer of the vessel wall, the *intima*, has the ability to retain lipoproteins

derived from circulating blood. A high plasma lipoprotein content leads to increased intimal uptake, likely through binding with enzymes and proteoglycans[13] on the surface of endothelial cells (ECs) that form the interior lining of the vessel[14]. Intimal accumulations of LDL cholesterol oxidize and activate resident macrophages by stimulation of scavenger receptors[15]. Macrophages engulf and opsonize the modified lipids. When cholesterol influx exceeds efflux, the cells become 'lipid laden'. This lipid loading of macrophages transforms them into cells with a foamy appearance, hence the name 'foam cells'. At predilection sites for atherosclerosis, or *atheroprone* regions, intimal accumulation of foam cells results in the formation of *fatty streaks*, macroscopically discernible areas with a high lipid content. Atheroprone regions have an impaired endothelial coverage and function[16], as well as a higher tendency to retain LDL particles. LDL cholesterol, when aggregated and oxidized in the arterial wall, is recognized and reacted upon by cells of the immune system, sparking a chronic inflammatory reaction[17].

Biomechanistically, atheroprone regions are characterized by a triad of properties: low shear stress, a high oscillatory shear index, and steep temporal and spatial gradients in shear stress[18]. Fluid shear stress in arteries is defined as the force inflicted on the vessel wall by the blood flowing parallel to it, and is dependent on blood viscosity and velocity. At sites exposed to a favorable shear stress, atherosclerosis rarely occurs. ECs directly exposed to the local shear stress, have important sensory functions for humoral as well as mechanic circulatory signals. Early atherosclerosis impairs the integrity of the EC layer, and the damage exposes the underlying cells, such as smooth muscle cells (SMCs), to the circulation[16].

During most of its development, atherosclerotic plaque does not cause any symptoms until the affected vessel becomes significantly narrowed (e.g. in *angina pectoris*) or occluded (e.g. in myocardial infarction). Slowly developing plaques may, through transitory periods of ischemia, induce the transformation of minor vessels into collaterals. This creates a natural bypass around the lesion[19].

The Atherosclerosis Council of the American Heart Association distinguishes six histological types of atherosclerotic lesions, only three of which are considered at risk to become clinically overt[20]. Throughout plaque development, the growing lipid core is separated from the vascular lumen by a collagen-rich fibrous cap (Fig. 1.1B), and symptoms generally occur when the fibrous cap gets thinner and ruptures (Fig. 1.1C). This exposes the highly thrombogenic lipid core to blood coagulant factors, which triggers thrombus formation. Thrombi cause ischemic symptoms, of which the severity depends on thrombus size; smaller thrombi may initiate a healing response similar to the process occurring in skin lesions, including the formation of a scar-like, protective fibrous cap *de novo*[21]. Plaques at risk of rupture (also called *vulnerable* or *unstable*) can with increasing accuracy be detected with imaging methods[22,23]. To induce a healing response in vulnerable plaques without prior thrombus formation, through local distribution of cellular triggers of e.g. collagen formation, is a novel therapeutic approach to transform a vulnerable into a stable plaque.

As an atherosclerotic plaque develops, calcium can accumulate in the intimal and medial layers. Advanced plaque calcification is characterized by the presence of cells and proteins found in bone, such as osteoblasts, osteoclasts, osteopontin and bone morphogenic proteins (BMPs), and can even give the plaque a trabecular bone aspect. There is an increasing scientific interest in mechanisms underlying arterial calcification, and how it might contribute to plaque stabilization or –vulnerability[24].

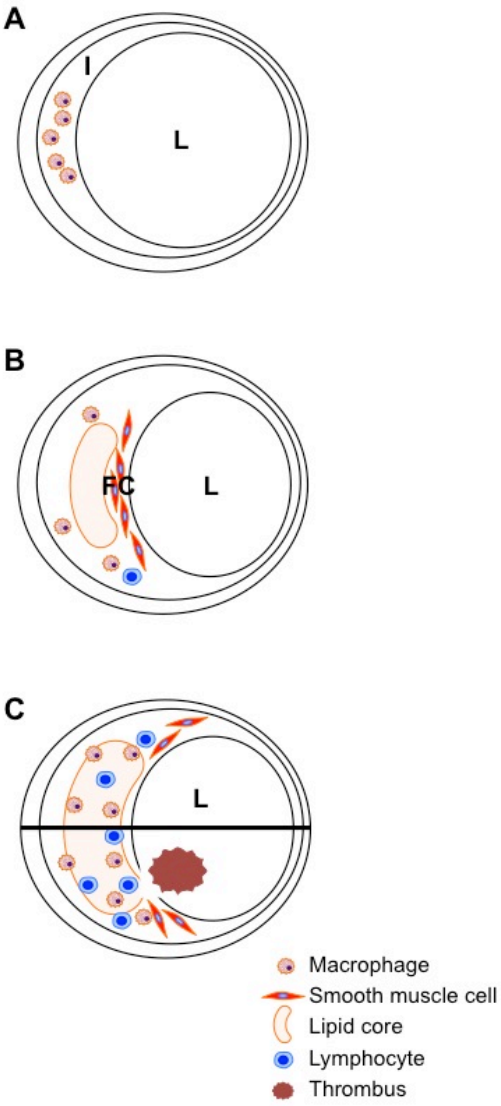

Figure 1.1. Arterial atherosclerosis starts with (**A**) thickening of the intima (*I*) and narrowing of the lumen (*L*) as plasma lipoproteins are incorporated. Macrophages invade the lesion and opsonize oxidized LDL cholesterol. (**B**) Smooth muscle cells enter the intima and produce extracellular matrix, covering the growing lesion with a collagenous fibrous cap (*FC*) and creating a stable lesion. Oxidized LDL cholesterol augments expression of adhesive molecules by endothelial cells lining the vessel, facilitating adhesion and invasion of lymphocytes. This initiates an inflammatory reaction. (**C**), Top: Vulnerable lesion with a thin fibrous cap, a large lipid core, and increased influx of lymphocytes. Bottom: Ruptured lesion containing a damaged fibrous cap and a lipid core exposed to the vessel lumen, attracting coagulation factors and initiating thrombus formation.

The most important producers of extracellular matrix in the fibrous cap are vascular smooth muscle cells (SMCs). SMCs form the medial layer of blood vessels. They originate from a variety of embryonic precursor cells distributed in a mosaic manner throughout the developing body. The boundaries of these embryonic cellular sources are poorly defined, making it difficult to map a single SMC to its individual origin[25]. In healthy arteries, the main function of SMCs is to maintain vessel tone and alter the luminal diameter, thereby regulating blood pressure. Under these physiologic conditions, SMCs are defined by expression of SMC-specific markers such as myocardin, smooth muscle cell myosin heavy chain (MYH11), smooth muscle α actin (ACTA2), and smoothelin (SMTN). The

transcription factor serum response factor (SRF), as well as the DNA modifying enzyme ten-eleven translocation 2 (TET2), have been shown to induce this contractile phenotype[26]. In response to certain pathologic stimuli, such as hypoxia, disturbed blood flow, injury, or inflammation (e.g. in atherosclerosis), SMCs downregulate these characteristic markers and adopt a phenotype characterized by proliferation, migration, and altered extracellular matrix production[27]. This phenotypic transition is partly driven by Krüppel-like factor 4 (KLF4), a transcription factor indicating stem cell-like capacities of cells[28], and platelet-derived growth factor (PDGF), a serum protein released by platelets and ECs which is strongly associated with cardiovascular disease[29]. Lipid accumulation in the plaque can even cause SMCs to adopt a macrophage-, almost foam cell-like phenotype, and as such stimulate progression of a plaque towards rupture[30,31]. Definitive analyses on whether these detrimental cell types derive from SMCs, macrophages, or other cell types, are still lacking; however, accumulating evidence suggests that these cells are indeed of SMC origin[28,32]. It is clear that induction of plaque stability involves the engagement of SMCs, although the appropriate signaling to and between these cells is required. This is one of many challenges in the development of targeted drugs and modifiers.

Chronic inflammatory diseases, cardiovascular disease included, are characterized by an imbalance between inflammation and resolution of inflammation. Resolution is the capability of an organism to regulate and dampen the inflammatory response by the release of resolvins, factors that actively promote restoration of tissue morphology and function[33]. The fact that certain inflammatory signals can activate resolution, and that their blockage can lead to aggravation of the disease, highlights the ineffectiveness of simply blocking one agent or pathway in the inflammatory cell machinery. MicroRNAs, acting in network patterns, have a proven capability to influence multiple targets in the same pathway. Modulating them has shown to successfully tip the balance in favor of resolution. Especially the cardiovascular system, a tightly regulated network of spatially distant organs (kidney, heart, liver, intestine), is an excellent target for microRNA therapy[34].

1.2 CARDIOVASCULAR DISEASES STUDIED

1.2.1 Abdominal aortic aneurysm

A relatively uncommon, but striking example of vascular disease is abdominal aortic aneurysm (AAA), defined as an expansion of the abdominal aorta (Fig. 1.2A) more than 1.5 times its size, or a diameter larger than 3 cm (Fig. 1.2B, left side). Risk factors for AAA largely overlap with those of atherosclerosis, with the exclusion of diabetes[35]. AAA cases seem to cluster within families, and although several genes have through genome-wide association studies (GWAS) been associated with the disease, no functional mechanism has yet been found to link any of these genes to the pathologic changes observed during the disease course[36]. An exception is the gene coding for cyclin-dependent kinase inhibitor 2B (CDKN2B)[37], a tumor suppressor gene with an inhibitory action on p53 signaling. As of today, no systemic treatment exists to halt AAA development; AAA (including ruptured AAA) can only be corrected by implantation of a polymeric graft inside the aorta, either

surgically or with an endovascular procedure (endovascular aortic repair, EVAR). Open surgical repair is an invasive abdominal operation, where the dilated vessel is relieved of mechanic strain while being left *in situ* (Fig. 1.2C). The introduction of EVAR reduced the invasiveness of AAA correction, but not all aneurysms lend themselves to be treated with EVAR[38].

During AAA development, inflammation of the aortic wall, decreased SMC content, and disruption of the extracellular matrix cause weakening and loss of elasticity[39]. In concert with specific biomechanical forces[40], these changes lead to a widening of the vessel. AAA typically remains asymptomatic until rupture occurs (Fig. 1.2B, right side), causing the aorta to drain directly in the abdominal cavity. Although a ruptured aneurysm may be temporarily 'contained' by the surrounding retroperitoneal tissue, it is generally considered that the chance of surviving an untreated AAA rupture is null. Despite recent treatment advances, partly due to the introduction of EVAR, the overall mortality of AAA rupture remains between 80-90%[41,42]. The combination of poor prognosis and absence of clinical symptoms, and the possibility to screen for AAA noninvasively with ultrasound, has led to the initiation of screening programs in about a dozen countries, with Sweden and Great Britain at the forefront[43]. Still, AAAs are typically discovered as an incidental finding during radiologic examination of other conditions[44]. Barriers in the implementation of AAA screening are cost and infrastructure[43], as well as the high number of AAA patients needed to be treated in order to prevent death due to rupture. The addition of identifiable circulating factors in the blood ('biomarkers') to current screening methods might provide a better prediction of rupture risk, thereby also increasing cost-effectiveness of AAA screening and leading to a better allocation of AAA repair surgery.

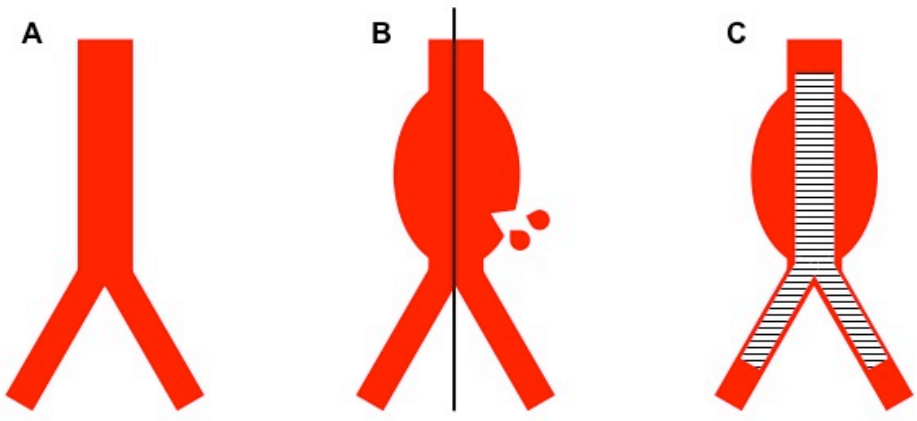

Figure 1.2. AAA is defined as an expansion of the normal aorta (**A**) creating a balloon effect (**B**, left side). When the weakened vessel wall yields to internal mechanical pressure, rupture occurs (**B**, right side). (**C**) With a polymeric implant, the aortic wall is relieved from direct strain, thus preventing or treating rupture.

1.2.2 Stroke

Stroke is recognized as a disturbance of cerebral function due to a vascular cause. Around 20% of strokes are caused by bleeding (hemorrhagic stroke), and 80% by occlusion

(ischemic stroke)[45]. In a cohort of 5017 patients with ischemic strokes, cardioembolism was the most common cause (25.6%), followed by carotid atherosclerosis (20.9%)[46]. In the context of this thesis, only the last form will be discussed.

Stroke caused by carotid atherosclerosis generally originates from a ruptured plaque around the carotid bifurcation. Thrombi dislodging from the formed clot travel to smaller arteries in the brain, where they become occlusive and produce symptoms such as temporary loss of vision (*amaurosis fugax*), transient neurological dysfunction (transient ischemic attack, TIA), or stroke. Stroke is an important cause of immediate death or severe disability including the need for lifelong care, reflected in the nearly 40 million disability adjusted life years lost to ischemic stroke[47].

Amaurosis fugax, TIAs or stroke are indications for carotid endarterectomy (CEA), a surgical procedure where the carotid artery is isolated, clamped, and opened to remove the plaque. Also eligible for this procedure are asymptomatic patients with a significant carotid stenosis[48], but in the majority of cases, the perioperative surgical risk of approximately 3% still outweighs the risk of plaque rupture[49,50]. New screening and imaging techniques allow for accurate detection of vulnerable plaque[22,23]. Ways to not only diagnose, but also stabilize such a lesion biologically rather than surgically, for example by stimulating the intrinsic healing response, would improve patients' perspectives and help clinicians make treatment decisions.

1.2.3 Radiation vasculopathy

The increasing efficacy of cancer treatment has led to a growing cohort of cancer survivors[51]. In these ex-patients, long-term adverse effects of cancer treatments are now becoming evident[52]. Radiation-induced vascular disease, or radiation vasculopathy, is an under-recognized problem in patients previously undergoing radiation therapy (RT) for cancer forms in the mediastinal and/or head and neck area (typically breast cancer, Hodgkin's lymphoma, or head and neck squamous cell carcinoma), where the heart and large conduit arteries are located[53,54]. With increasing dosage, local RT increases the risk for ischemic heart disease[55,56]. In patients receiving RT for head and neck squamous cell carcinoma, carotid artery irradiation is a significant risk factor for carotid occlusive disease[57-59].

The underlying cause of radiation vasculopathy is radiation-induced arterial inflammation, characterized by the expression of inflammatory mediators in the nuclear factor kappa B (NF-κB) pathway[60]. This NF-κB activation is chronic and detrimental, and is not only suspected to induce a more aggressive form of atherosclerosis; it also causes thrombosis and subsequent failure of non-vascularized transplanted tissue[61,62]. As the survival rates of cancer patients improve, there is a need for novel therapies to halt the vascular inflammatory reaction if the goal is to aim for a *healthy*, rather than merely *cancer-free* life for cancer survivors.

1.3 NON-CODING RNA

The 'central dogma of molecular biology', first described by Watson and Crick in 1953, states that there is a unidirectional flow of genetic information from the DNA. Via transcription of messenger RNA (mRNA), information is translated into proteins, which regulate cellular function. That there might be exceptions to this rule, has been proposed less than a decade later[63], but was not further investigated until the discovery of 'bacterial interfering RNA' in 1984[64]. Describing this phenomenon for the first time in an animal model, Lee *et al* sparked the interest in the biological functions of RNA interference (RNAi)[65]. Gradually, all the genetic information not directly encoding proteins, previously called 'junk DNA', is now being mapped and showing that not 1-3%, but actually approximately 80%, of our DNA sequence is potentially functional[66,67].

A variety of functional elements (hereafter non-coding RNA, ncRNA) are actively transcribed from the human DNA, and interfere with cellular processes at many levels, thereby continuously challenging current knowledge about gene function[68]. Present-day literature distinguishes between a multitude of ncRNA subclasses, depending on function, size, structure and genomic location[68,69]. Almost every newly discovered ncRNA has a different physiological role, which makes generalization of ncRNA function difficult[70,71]. ncRNAs can be sub-classified according to function, which can be structural (transfer-RNAs, ribosomal RNAs, small nucleolar RNAs) or regulatory (microRNAs, piwi-interacting RNAs, small interfering RNAs). For ncRNAs with as of yet unknown function, sub-classifications can be made based on structure (circular RNAs) and location (long intergenic RNAs, enhancer RNAs), but the most common cutoff used is length (larger or smaller than 200 nucleotides). Interestingly, the number of non-coding transcripts increases exponentially with species complexity, where coding transcripts seem to asymptote around 30,000[72]. ncRNAs can therefore be viewed as 'adapters', providing specificity to the molecule-to-molecule communication inside cells: information needed to perform complex biological tasks. This implies that ncRNAs can give information about the temporal and developmental state of a cell, in an even more specific manner than proteins or mRNAs[73]. A disadvantage to this specificity is that ncRNAs typically are fewer in number and more transiently expressed, and therefore less easily detectable. This technological challenge can be overcome to a certain extent by deep sequencing techniques such as RNA-sequencing (RNA-Seq), but this generates enormous amounts of transcriptomic information[74]. The extraction of biologically meaningful elements, the assessment of their contribution to disease, and the definition of their therapeutic potential, requires a combination of bioinformatics, chemistry, cellular biology, and medicine. When determining therapeutic interest, an important selection criterion for certain ncRNAs, especially miRNAs, is conservation among species[75]. The reasons for this are two-fold: conservation gives an indication of the relevance of ncRNAs, and allows for experimentation *in vivo*. That a ncRNA is not conserved, however, does not necessarily indicate its redundancy. This seems to be the case particularly for long noncoding RNAs (lncRNAs)[76].

1.3.1 MicroRNAs

MicroRNAs (miRNAs) are 20-to-22-nucleotide-long single-stranded ncRNA molecules that are able to base pair with homologous 8-nucleotide-long seed sequences within target mRNA molecules. miRNAs generally reside in the cell cytoplasm, coupled to RNA-binding protein Argonaute 2 (AGO2). Together with the enzyme Dicer, AGO2 forms the active part of the RNA-induced silencing complex (RISC), responsible for RNA-induced silencing or RNAi[77]. RISC activation induces mRNA deadenylation and decay, or other types of post-transcriptional regulation. The net effect is, typically, repression of protein translation (Fig. 1.4). Because one miRNA can have multiple mRNA targets, miRNAs are able to influence several steps in biological processes, e.g. signaling cascades, making them powerful modulators of these processes[78]. Genomic variants affecting miRNA:mRNA pairing can influence miRNA-mediated gene expression regulation[79]. Thus, data from genome-wide association studies can be combined with information about miRNA functionality, in order to find miRNAs involved in, for example, cardiovascular disease risk[80]. These discoveries are generally made *in silico* and, therefore, merely explorative. Whether the identified variants indeed do confer an altered disease risk always needs to be verified experimentally.

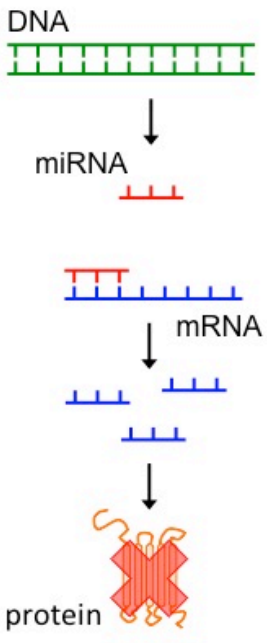

Figure 1.4. miRNAs base pair with mRNA, inducing its de-adenylation and decay. The net effect is inhibition of protein translation.

miRNA nomenclature is based on the order of their discovery. The first known miRNAs, lin-4 and let-7, are logical exceptions to this rule[65]. To date more than 28,000 miRNA transcripts have been identified[81]. Typically, miRNAs are annotated starting with the species they can be found in (e.g. *homo sapiens*, hsa; *mus musculus*, mmu), followed by a number, and a suffix -3p (indicating the 3' strand derived from the stem-loop precursor) or -5p (the 5' strand). Since the ncRNA field is developing rapidly, nomenclature rules are subject to change; some publications still refer to the 3' strand as the 'star' (*) strand, based on the assumption that this 'passenger' strand has less biological meaning than the 'original' miRNA. As many 3' miRNAs turned out to have important functions and could by no means be considered inferior to their 5' counterpart, the 'star' annotation was abandoned. miRNAs differing only in 1 or 2 nucleotides, often with largely the same gene targets, are commonly grouped and receive the suffix -a, -b, -c, etc. Most well-studied miRNA sequences are conserved among species, and therefore a commonly used approach to explore miRNA function is to generate a knockout animal. This approach has the additional advantage that in physiological conditions, most miRNAs seem to be redundant. miRNA knockout mice generally do not have a striking phenotype, and modulation of miRNAs with artificial inhibitors (anti-miRs or antagomiRs) or enhancers (miRNA mimics or pre-miRs) is well tolerated under normal conditions *in vitro* and *in vivo*. However, when cells or organisms are

stressed, e.g. in disease states or during starvation, miRNAs limit the amount of mRNA escaping excess transcription. In this way they contribute to stabilizing a cell's physiological state, hence the popular annotation of miRNAs as 'fine tuners' of cell output[78] and sustainers of a bi-stable or oscillatory cellular state[82]. Dependent on cell type, disease progression, and other physiologic parameters, miRNA interference can be beneficial or detrimental, and therapeutic interest exists for anti-miRs as well as pre-miRs.

Apart from their intracellular functions, miRNAs can be detected in the circulation, and are hypothesized to take part in information exchange between cells in an organism. Complete fields of research are dedicated to determining what type of subcellular carriers are responsible for this intercellular communication, and their putative modes of action[83,84]. It is likely that the type of carrier, and the extent to which a specific miRNA is involved in this system, is cell-specific. By using qRT-PCR, deregulated levels of miRNAs can be reproducibly detected and quantified[85]. miRNAs measured in the circulation have been shown to contribute to estimating the risk of cancer[86] myocardial infarction[87], and AAA progression[88]. There are, however, caveats to this approach. Medication can be an important confounding factor when measuring levels of circulating miRNAs, as certain drugs are known to hamper the expression of some cell-specific miRNAs[85]. Awareness of and correction for confounders, as well as uniform sampling and sample processing, are therefore imperative in miRNA research.

1.3.2 Therapeutic applications of non-coding RNAs

Besides their diagnostic function, perturbations in ncRNA expression can be altered with specific modulators, in order to change the disease course. An example of successful ncRNA therapy is blockage of miR-122, which in patients with hepatitis C virus can reduce viral titers to undetectable[89]. Despite the potential of RNAi therapy, there is a discrepancy between the number of ncRNAs shown to be of therapeutic interest based on *in silico* and *in vitro* studies, and those being investigated as therapeutic targets *in vivo*. With the exception of organs and tissues amenable for topical therapy – the eye, skin, and local tumors – it is technically difficult to direct ncRNA-modifying drugs to a target organ, or cell, without affecting other cells. The currently most widely used approach to envelope and deliver ncRNA modulators is with nanoparticles and liposomal carriers. In this context, it is not surprising that liver disease emerged as the first successful therapeutic application of anti-miRs[89] given the affinity of hepatocytes for particles with liposomal size and structure[90]. Several other chemical compounds have been investigated as candidate vehicles[91] in this rapidly developing field.

Therapeutic modulation of miRNA function involves inhibition as well as stimulation. Inhibition of miRNAs generally means inactivation, and because of the fact that miRNAs operate in the cytoplasm, anti-miRs need only be delivered to the cellular periphery. By constructing oligonucleotides with an artificially added bonding bridge in the ribose moiety of the nucleotide, it is possible to create a molecule able to irreversibly bind to miRNA. This 'locked nucleic acid' (LNA) technique produces a double-stranded RNA molecule, which is degraded by the cell. The most successful example of LNA-mediated miRNA inhibition is

the aforementioned miR-122-antagonizing therapy, applied in hepatitis C virus patients. Since miR-122 is essential for the virus to replicate, anti-miR-122 rendered patients virus-free[89]. Introducing miRNA mimics is more challenging, because a cell needs to process the mimic construct such that it is incorporated in the RISC. This requires introduction into the nucleus, and to achieve this, more advanced chemical modifications are needed.

For administration of nucleic acid-modifying therapies *in vivo*, including miRNA mimics, the most clinically advanced techniques are virus-based. Adenovirus[92], adeno-associated virus[93], and retroviruses[94] are approved by regulatory agencies and used in clinical trials. We successfully used a lentiviral vector to administer pre- and anti-miR-24 in mouse AAA development (Paper I). Viral vectors are an effective, yet controversial, delivery method. A primary concern is the propensity of adenoviral particles to trigger an immunogenic response, thereby causing adverse symptoms or inactivating their therapeutic effects. Immunosuppressive therapy can dampen this unwanted immunogenicity, but may expose a patient to opportunistic infectious diseases. For retroviral vectors, integration of the viral genetic material into the host DNA is a major issue. Retroviruses tend to integrate genetic material into promoter regions. This has shown to be deleterious in a clinical trial, where pediatric patients, treated for immunodeficiency, developed leukemia[95]. Other issues such as vector mobility, where virus particles travel beyond the area of interest, and high production costs, have initiated a search for non-viral strategies[96].

An efficient, commercially available non-viral vector is polyethyleneimine (PEI). PEI is a strongly cationic polymer, which can be used to package nucleic acids into positively charged particles. When presented to the negatively charged cell surface, PEI particles undergo endocytosis and, once in the cytosol, render the nucleic acid free to enter the nucleus[97]. PEI-delivered miRNA mimics injected systemically reach a variety of organs[98], including blood vessels[99], without eliciting a major immune response. We successfully used PEI to deliver mimics of miR-210 and miR-29b to murine SMCs in carotid plaque (Paper III) and radiation vasculopathy (Paper IV).

There are disadvantages to the use of PEI, for example its direct cytotoxicity[100,101]. For ncRNA-modulating technologies in general, the aim is to minimalize complications such as unwanted reactions, side effects, hypersensitivity, inadequate concentration at target locations and potentially rapid clearance of the therapeutic substance. Non-systemic, i.e. local, administration techniques are gaining interest[96]. In vascular disease, drug-eluting stents have emerged as potential ncRNA carriers: In a humanized rat model, an anti-miR-21 eluting stent increased the local availability of the miRNA modulator whilst avoiding toxicity in other organs, which indeed was a problem with systemic treatment[102]. More revolutionary delivery techniques include drug-containing microbubbles burst by ultrasound waves[103] or 'sonoporation' (ultrasound-triggered opening of membrane pores) to deliver e.g. PEI[104]. Whether these techniques will finally reach the clinic, depends largely on their efficacy in (pre)clinical studies. An important translational step is the transition from mice to larger animal models such as pigs, which have a greater similarity to humans both in size and

taxonomy[105] and can be genetically modified to develop cardiovascular disease[106]. Our group is at the forefront of these developments.

1.4 NON-CODING RNAS IN CARDIOVASCULAR DISEASE

The cardiovascular system is a strictly regulated network sensitive to small perturbations. The functional characteristics of ncRNAs as regulators of cell fate make them particularly suited to control vascular development and homeostasis in physiological conditions. Their role in cardiovascular disease has also been well established[107].

1.4.1 Non-coding RNAs in vascular smooth muscle cell biology

Perhaps one of the best-described miRNAs inducing a proliferative vascular smooth muscle cell phenotype is **miR-21**. In cancer, miR-21 is characterized as 'onco-miR', promoting cancer cell proliferation and metastasis. Among miR-21 targets are the tumor suppressor genes B-cell CLL/lymphoma 2 (BCL2), phosphatase and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4). In vascular smooth muscle cells, miR-21 induces a dedifferentiated, proliferative phenotype, which contributes to e.g. in-stent restenosis in percutaneous coronary interventions (PCI). Anti-miR-21 therapy was able to prevent SMC proliferation and reduce in-stent restenosis[102]. That SMC dedifferentiation is not necessarily detrimental was shown in animal models of AAA. miR-21 overexpression prevented AAA rupture and expansion, due to a proliferative, anti-apoptotic effect via inhibition of PTEN in SMCs[108]. In atherosclerotic plaque development, miR-21 could also have stabilizing effects by increasing the number of SMCs in the fibrous cap.

The **miR-143/145** cluster is highly expressed in SMCs and was one of the first miRNA groups shown to induce SMC contractility and counteract pathological changes by targeting *KLF4* mRNA[109]. Besides KLF4, miR-143/145 target an array of proliferative factors including platelet-derived growth factor (PDGF) and angiotensin-converting enzyme (ACE).

In AAA and atherosclerosis, dedifferentiated vascular smooth muscle cells can contribute to stabilizing the vessel wall by producing extracellular matrix. The **miR-29** family consists of three members, miR-29a, -b and -c. Together, these miRNAs target at least 16 genes related to extracellular matrix production[110]. Higher expression of miR-29 is associated with aneurysm expansion in humans and mice, and inhibition of miR-29 family members effectively prevents aneurysm expansion in mice[111,112] and affects vascular wall strength and elasticity[113]. In tissues such as the lungs[114] and heart[115], miR-29b regulates fibrosis. Indeed, when expanding the view on fibrotic diseases, and including other organs such as the kidney, a broad interest in ncRNA involvement can be seen. Two lncRNAs, **np_5318** and **np_17856**, have been functionally linked to progressive kidney injury[116]. Under hyperglycemic conditions, kidney cells upregulate expression of **miR-1207-5p** and its host gene, lncRNA plasmacytoma variant translocation 1 (**PVT1**)[117]. Because all of these ncRNAs operate through the same fibrosis-related cellular mechanistic pathways, like miR-29, it is likely that they also have an impact on vascular disease.

PDGF release stimulates expression of the cluster **miR-221/222**, promoting a proliferative phenotype in SMCs via targeting of the cell-cycle inhibitors cyclin-dependent kinase inhibitor 1B (CDKN1B) and –1C (CDKN1C). The stem cell factor KIT, responsible for myocardin transcription, also belongs to the miR-221/222 targets[30], further strengthening the SMC dedifferentiating function of these miRNAs. Angiotensin II (ANGII), mediator of hypertension and a well-known culprit factor in cardiovascular disease, induces expression of several lncRNAs. In rat cells, one of these ANGII-responsive lncRNAs, **Lnc-Ang362** or MIR222HG, is a host gene for miR-221/222, meaning that the lncRNA and miRNA cluster are co-transcribed in these cells[118]. This example illustrates how tightly interwoven the network of ncRNA regulation can be.

RNA-Seq studies in human vascular cells uncovered Smooth muscle and Endothelial cel-enriched migration/differentiation-associated long Non-Coding RNA (**SENCR**) as a lncRNA involved in the contractile gene program of SMCs. Knockdown of this lncRNA showed an increase in SMC motility and loss of contractile proteins, suggesting a role for SENCR in the maintenance of a 'normal', differentiated SMC phenotype[119].

H19 is a maternally imprinted gene mostly expressed during embryonic development[120]. Most studies suggest a growth-promoting effect of H19, for example in skeletal muscle[121] and myogenic progenitor cells[122], but also cancer cells[123,124]. After vascular injury, phenotypic changes of SMCs have been associated with increased expression of H19 in rats, possibly displaying a role of H19 in neointima formation[125].

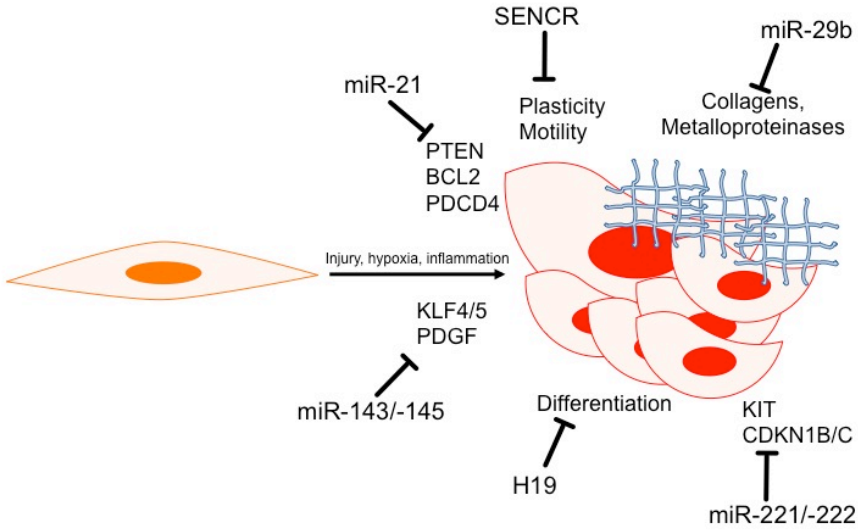

Figure 1.5. SMCs respond to pathologic stimuli by dedifferentiation, characterized by proliferation and extracellular matrix production. ncRNAs affect this process through many biological pathways.

1.4.2 Non-coding RNA in vascular endothelial cell biology

Through its demonstrated effects on vascular endothelial cells (ECs), **miR-126** is well known as a regulator of vascular integrity, both during embryonic development[126] and in atherosclerosis[127]. Circulating miR-126 expression has been associated with a reduced cardiovascular risk, which suggests an important role for microvesicles as miRNA

carriers[128]. In a carotid re-endothelialization model, nanoparticle-mediated delivery of pro-angiogenic miR-126 improved endothelial repair[129]. miR-126 has also shown to be abundant in platelets, a finding that challenged the endothelial origin in the context of changes in circulating miR-126 levels, and which also suggested antiplatelet treatment as a confounder for deregulated miR-126 levels. This stresses the importance of a proper patient control selection and the use of a co-expression network of miRNAs, rather than one miRNA, when exploring the diagnostic potential of miRNAs.

Other miRNAs associated with EC (patho-)physiology are the **miR-17≈92a** cluster and **miR-23≈27≈24**. Members of the miR-17≈92a cluster include miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1, and target EC functions such as angiogenesis, sprouting and vascular remodeling via sirtuin 1 (SIRT1), integrin subunit alpha 5 (ITGA5), Janus kinase 1 (JAK1), sprouty protein 2 (SPROUTY2), and semaphorin-6A (SEMA6A)[130-132].

'Myocardial infarction-associated transcript', **MIAT**, is an example of a long non-coding RNA whose expression is correlated with cardiovascular risk. At approximately 9 kilobases, the transcript is substantially larger than a miRNA, but does not encode any translational product. Overexpression- and inhibition studies *in vitro* and *in vivo* have thus far shown effects of MIAT in a range of cell types and disease conditions, from schizophrenia to diabetic retinopathy[133]. The latter association of MIAT with microvascular dysfunction was attributed to competing effects of MIAT and miR-150-5p on VEGF, with MIAT functioning as a miRNA sponge alleviating the miR-150-5p inhibition on VEGF[134]. Unpublished work from our group suggests a key role for MIAT not only in endothelial cells, but also for smooth muscle cells during atherosclerotic plaque stabilization processes (Li et al, unpublished).

A long non-coding RNA with a more clearly elucidated function in endothelial dysfunction and diabetes is 'metastasis associated lung adenocarcinoma transcript' **MALAT1**. MALAT1 is highly conserved among mammals, is localized in the nucleus and controls functional levels of serine/arginine splicing factors, thereby regulating alternative splicing[135]. In endothelial cells, MALAT1 is induced upon hypoxic stress, and its effects are pro-proliferative and anti-apoptotic[136]. Because of its effects on endothelial cells, a role in diabetic nephropathy and overall endothelial function has been attributed to MALAT1[137].

In human endothelial cells subjected to hypoxia, two long intergenic RNAs (**LINC00323** and **MIR503HG**) are induced and have been linked to angiogenic capacity. Modulation of these ncRNAs could have impact on tissue vascularization and recovery after ischemia[138].

1.4.3 Non-coding RNA in vascular inflammation

In autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus, **miR-155** is induced. An increase miR-155 expression was found to be downstream of pro-inflammatory stimuli such as tumor necrosis factor (TNF), Toll-like receptors (TLRs), and NF-κB signaling and it has been demonstrated to be involved in regulation of B and T cell responses, as well as innate immunity[139]. Inhibition of miR-155

has been shown to dampen rheumatoid arthritis in mice[140]. In a cardiovascular context, miR-155 has been studied extensively with regard to its role in inflammation, sustaining and aggravating atherosclerosis, indicating atheroprotective[141] as well as detrimental[142] effects of this miRNA.

The two members of the **miR-146** family, miR-146a and miR-146b, are induced by inflammatory components of the NF-κB pathway[143]. Both miRNAs have an identical seed sequence, which targets the 3' UTR of many genes in the same pathway, such as interleukin 1 receptor associated kinase 1 (IRAK1), nuclear factor kappa B subunit 1 (NFKB1), Toll-like receptor 4 (TLR4), and TNF receptor associated factor 6 (TRAF6)[144]. Enforced miR-146b expression in monocytes leads to a reduction in at least seven inflammatory cytokines[145,146], supporting the notion that these miRNAs might be part of a feedback loop limiting inflammation. In human atherosclerotic plaque, miR-146a and -b are upregulated[147], and miR-146b-5p is known to promote platelet-derived growth factor-BB (PDGF-BB)-induced SMC proliferation and migration[148], which ay contribute to a stable plaque phenotype.

miR-150 is a key regulator of immune cell differentiation and activation[149,150]. In patients with critical illness and sepsis, its expression is predictive for survival[151]. **miR-181** is expressed in vascular- as well as immune cells, and is critically involved in vascular inflammation through its effects on the NF-κB signaling pathway[152]. Human monocytes stimulated with the immune response-trigger lipopolysacchararide (LPS) upregulate the ncRNAs **IL1β-eRNA** and **IL1β-RBT46(+)**, both containing significant enrichment for NF-κB binding sites[153].

1.5 BIOMARKERS

By providing information about ongoing cellular processes, biomarkers can help to diagnose and manage cardiovascular disease. A biomarker is a compound released by cells or tissues, and which can be detected in order to give information about the presence and progression of disease, as well as determine which treatment will most likely benefit the patient (Fig. 1.6). An ideal biomarker can be found non-invasively (e.g. in blood plasma, urine, or saliva), is sensitive and specific, has a fast and cheap detection method, and detects a treatable condition[154].

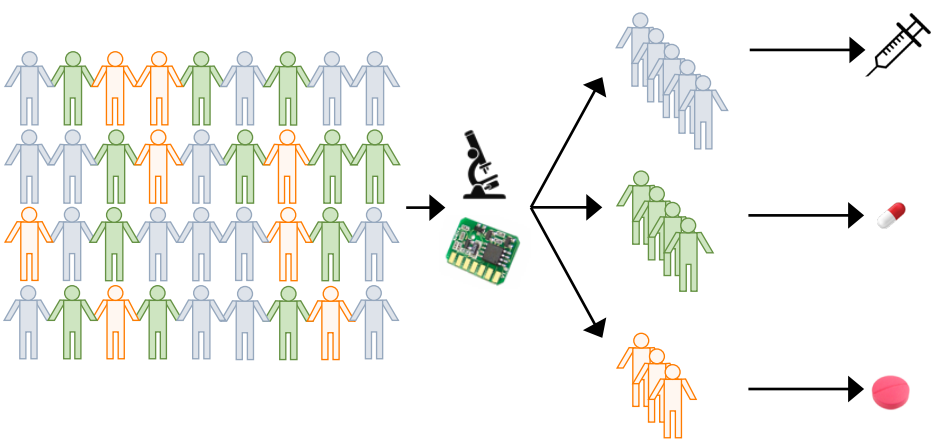

Figure 1.6. Biomarkers can indicate which patient will most likely benefit from which treatment.

ncRNAs, especially miRNAs, are attractive disease biomarkers. Whereas 'naked' RNA is rapidly degraded in the circulation, miRNAs can be stable in peripheral blood, but also under unfavorable handling conditions such as room temperature– and repeated freeze-thaw cycles[155]. There are even reports that propose a role for miRNA in forensics[156] because of their stability in stored specimens. The stability of miRNAs is often attributed to their folding, as well as the tendency of cells to release these molecules in subcellular envelopes such as microvesicles, exosomes and apoptotic bodies, which provide a barrier against degradation. Other subcellular carriers of miRNAs reported are HDL molecules and proteins such as argonaute II (AGO2)[84]. There even are reports that miRNA-carrying vesicles released by cells can be taken up by recipient cells in a paracrine manner, or even by distant organs, indicating that this vesicular miRNA transport is targeted, rather than random[157]. Many questions remain to be answered in extracellular vesicle research, which on its own encompasses an entire expanding research field[83].

Another advantage of miRNAs as biomarkers is their sensitivity and specificity[158], given that certain miRNAs are tissue-specific (e.g. miR-208 for myocytes), and so-called 'co-expression networks', indicating a panel of deregulated miRNAs representing a disease phenotype, can provide a detailed 'signature' of a disease and its progression. They can easily be quantified by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

A more recently discovered class of ncRNAs, circular RNA (circRNA), possesses intrinsic stability. circRNAs are ncRNAs joined at their 3'-5' ends by a process named 'backsplicing', thus lacking sequence 'exposed' to endonucleases, a structural feature rendering them exceptionally stable[159]. Unraveling of the function of circRNA has caught on in many research fields, with some reports showing that they can serve as 'sponges' for miRNAs, thereby suppressing miRNA activity[160]. Future studies might lead to the conclusion that the biomarker potential of circRNAs, due to their stability and cell specificity, equals – if not exceeds – that of miRNAs as well as other ncRNA species.

2 THE PRESENT STUDY

2.1 AIMS

The general aims of this thesis are to define which non-coding RNAs play a role in vascular remodeling and atherosclerotic plaque formation, and how modification of non-coding RNAs can contribute to inhibiting these pathologic processes.

The specific aims per paper are as follows:

Paper I To find miRNAs associated with murine and human AAA development and progression, and to investigate whether modification of these miRNAs can mitigate AAA.

Paper II To identify miRNAs that can be used as plasma biomarker for AAA development and rupture.

Paper III To investigate miRNAs of clinical interest in patients with carotid stenosis at risk of stroke, and to see if modulation of these miRNAs can prevent carotid plaque rupture.

Paper IV To investigate which vascular disease-related miRNAs are affected in radiation-induced vasculopathy, and if modification of these miRNAs can dampen the vascular inflammatory reaction.

2.2 METHODS

This section includes a summary of methods used in the various projects. Detailed method descriptions can be found in the respective manuscripts.

2.2.1 Human biobank material

All Swedish studies were approved by the Ethical Committee of Northern Stockholm. SMART was approved by the University Hospital Utrecht ethics committee. For all studies, patient informed consent was obtained according to the Declaration of Helsinki.

2.2.2 Abdominal aortic aneurysm disease

2.2.2.1 FAD

Patients referred from 2006-2014 to the Department for Vascular Surgery of the Karolinska University Hospital for open or endovascular repair of an AAA were included in the study. The study was supported by EU grants under the 7th Framework Programme Fighting Aneurysm Disease (FAD, project ID 200674). Control blood plasma donors were recruited from patients referred to the Vascular Surgery department for non-AAA atherosclerosis-related symptoms. Informed consent was obtained from all patients. Blood was sampled during preoperative consultation and processed according to standard procedures for separation of the mononuclear cell fraction and RNA isolation. During open AAA repair surgery, biopsies of the aortic wall were sampled, and stored at -80°C until RNA isolation.

2.2.2.2 SMART

Rationale and design of the SMART study cohort, as well as abdominal aortic aneurysm (AAA) definition and policy, have been described in detail before[161,162]. Briefly, patients aged 18-80 with clinically manifest vascular disease or cardiovascular risk factors, referred to the University Medical Center Utrecht, The Netherlands, were enrolled. Patients received a standardized vascular screening including a health questionnaire, laboratory assessment, and ultrasonography. For this study, we included 200 SMART patients with ultrasonographically defined AAA, i.e. aortic diameter >30 mm, and 200 age-, gender-, CVD risk profile- and medication-matched non-AAA SMART control patients. Follow-up ultrasound examinations were conducted annually for AAAs between 30-39 mm; AAAs 40-55 mm were followed up biannually. AAAs >55 mm were considered an indication for surgical repair. The treatment policy was a recommendation; final treatment decision was subsequently made between treating specialists and patients. From patients who were eligible for AAA repair, additional blood was sampled 6 months after surgery.

2.2.3 Carotid atherosclerotic disease: BiKE

Patients undergoing surgery for symptomatic or asymptomatic high-grade (>50% NASCET) carotid stenosis at the Department for Vascular Surgery, Karolinska University Hospital, were consecutively enrolled in the Biobank of Karolinska Endarterectomies (BiKE) and clinical data recorded on admission. The BiKE study cohort demographics, details of sample

processing including control (normal artery) samples have been previously described[163]. Briefly, symptoms of plaque instability were defined as transient ischemic attack (TIA), minor stroke or *amaurosis fugax*. Patients without qualifying symptoms within six months prior to surgery were categorized as asymptomatic and indication for carotid endarterectomy (CEA) based on results from the Asymptomatic Carotid Surgery Trial (ACST)[49]. Patients with known atrial fibrillation were excluded. For this study, carotid endarterectomy tissue and blood samples from *n*=7 symptomatic and *n*=5 asymptomatic subjects were collected during surgery. We validated our findings in local plasma samples in a separate BiKE validation cohort of 7 versus 7 patients. During clamping of the carotid artery for 5 minutes before opening of the vessel, blood from the lesion site was sampled in EDTA-containing tubes. The resultant plasma samples were stored at -80°C before further processing. Hemolytic plasma samples were excluded from miRNA analysis. All studies were approved by the Ethical Committee of Northern Stockholm; patient informed consent was obtained according to the Declaration of Helsinki.

2.2.4 Radiation vasculopathy

We have analyzed samples from Karolinska University Hospital's Biobank of Irradiated Tissues at Karolinska (BIRKA). 15 pairs of arterial biopsies were harvested during head and neck cancer reconstruction with microvascular free tissue transfer in 15 preoperatively radiated patients. Before performing microvascular anastomosis, biopsies were harvested from the radiated cervical artery (recipient site) and from the non-radiated (donor) artery of the transferred autologous tissue. Biopsies were dissected from surrounding tissue and surgical material under a dissection microscope, where care was taken not to damage the endothelium. Vascular tissue was frozen and stored at -80°C until RNA extraction. For immunohistochemical analysis, tissue from a paired subset of three patients was fixed in 10% formalin and paraffin-embedded.

2.2.5 Murine models

The use of mice as a model to study atherosclerosis has advantages as well as disadvantages. Their relatively low cost, small size, and the availability of well-characterized, inbred strains make them a popular animal model. Disadvantages are the immunological differences between mouse and human[164], and genetic dissimilarity[165]. Porcine models are a logical next step in the translation of basic vascular biological principles and treatment options into clinical applications. In ncRNA research, this step has been taken for in-stent restenosis[166] and ischemia/reperfusion injury[167], and our current efforts include a miRNA profile of porcine aneurysm expansion (unpublished).

The by far most important disadvantage of mouse- as well as pig models in cardiovascular research is their complete (mice) or advanced (pigs) resistance to atherosclerosis. For cardiovascular research, investigators resort to genetically modified, high-cholesterol animals, such as low-density lipoprotein receptor- (*Ldlr*$^{-/-}$), and apolipoprotein E (*Apoe*$^{-/-}$) knockout mice, and *LDLR*$^{-/-}$ pigs[106]. Alternatively, or in combination with genetic knockout,

surgical and chemical manipulation and diet-induced metabolic disruption are methods we used to generate cardiovascular disease phenotypes.

2.2.5.1 Angiotensin II infusion

As a model of AAA formation and rupture, we used angiotensin II (ANGII) infusion in male *Apoe*$^{-/-}$ mice, first described by Daugherty *et al*[168]. Briefly, osmotic minipumps releasing ANGII at 1,000 ng/kg min^{-1} were placed under 2% isoflurane anesthesia. This ANGII dosage is reported to increase blood pressure by approximately 25 mmHg. In this model, AAA formation however does not seem to be caused by hypertension, since dampening as well as complete prevention of AAA formation has been achieved without blood pressure lowering[108,169]. At the pathological basis of AAA formation in this model is the mass influx of macrophages to the vascular medial layer and associated elastin degradation[170]. Using ultrasound (Vevo 2100, Visualsonics, Toronto, Canada), we measured the maximum infrarenal aortic diameter at baseline and at 7, 14, and 28 days after pump implantation. On day 7, blood was sampled in EDTA containers and centrifuged at 10,000×G for 10 minutes. On day 28, mice were sacrificed through CO_2 inhalation, exsanguinated by heart puncture, and perfused with 4°C PBS before organ harvesting. Aortas were embedded in OCT compound (Histolab, Gothenburg, Sweden), or snap-frozen, and stored at -80°C.

2.2.5.2 Isolating peritoneal mouse macrophages

Mice were euthanized by CO_2, 4 days after injection of Brewer's thioglycollate (Sigma-Aldrich, St. Louis, MO, USA). A quantity of 5 mL of 4°C phosphate-buffered saline (PBS) was injected twice into the peritoneal cavity of *Apoe*$^{-/-}$ mice with or without ANGII-induced AAA. Macrophages were then withdrawn from the intraperitoneal cavity and put into suspension, centrifuged at 1,500 rpm for 5 min at 4°C and 5 mL of RBC lysis buffer was added to the pellet. After incubation and centrifugation, 1 mL of 37°C RPMI 1640, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PEST, all Gibco, Thermo Fisher Scientific) per mouse were added to the pellet. After counting and dilution (10-fold), cells were plated, analyzed, and used for further experiments.

2.2.5.3 Porcine pancreatic elastase infusion

The porcine pancreatic elastase infusion model (PPE) as an *in vivo* approach to infrarenal aneurysm formation was first described in rats by Anidjar *et al*[171] and adapted for mice by Pyo *et al*[172]. We used a modified version of the mouse PPE model as visually presented by Azuma *et al*[173]. Briefly, male C57/BL6 mice (Charles River, Wilmington, MA, USA) were anesthetized with 2% isoflurane. The abdominal cavity was accessed through median laparotomy and the retroperitoneum exposed with retractors, leaving the intestines *in situ*. After opening of the retroperitoneum, the aorta and inferior vena cava were separated from the level of the left renal vein to the aortic bifurcation. All aortic branches within 1 cm of the bifurcation were temporarily ligated with 6.0 silk ligatures (Vömel, Kronberg, Germany). After placement of proximal and distal temporary silk sutures, aortotomy was performed with the tip of a 30-gauge needle. Through the aortotomy, a heat-tapered PE-10 polyethene

catheter was introduced and secured with a silk ligature. The isolated aortic segment was infused with PPE solution (1.5 U/mL in PBS; Worthington Biochemical Corporation, Lakewood, NJ, USA) under sufficient pressure to achieve a 50-70% aortic expansion for the duration of 8 minutes; control mice received PBS. After removal of the catheter, the aortic segment was flushed with saline and the aortotomy closed with a 10.0 polyamide monofilament suture (B Braun, Melsungen, Germany). Temporary ligations were removed and the abdominal wall closed with 5.0 Vicryl sutures (Ethicon, Somerville, NJ, USA), followed by closure of the skin. Mice received 0.1 mg/kg buprenorphine as post-operative analgesic. At 7, 14 and 28 days after PPE infusion, aortic aneurysm size was assessed by ultrasound (Vevo 2100, Visualsonics, Toronto, Canada), measuring maximum infrarenal aortic diameter. Four weeks after surgery, animals were sacrificed with CO_2 inhalation, exsanguinated by heart puncture, and perfused with 4°C PBS before organ harvesting. Aortas were embedded in OCT compound (Histolab, Gothenburg, Sweden), or snap-frozen, and stored at -80°C.

2.2.5.4 Carotid ligation – cast

Male, 12-week old *Apoe*$^{-/-}$ mice (Taconic, Hudson, NY, USA) were used for this experiment. *Ad libitum* access to water and food (standard chow diet) was ensured. Animals were anesthetized with 2.0% isoflurane-containing oxygenated air for the duration of surgical procedures. Depth of anesthesia was verified by toe pinching. Via medial neck incision, the right carotid artery was separated from the surrounding connective tissue. A 5.0 Vicryl suture (Ethicon, Somerville, NJ, USA) was placed around the common carotid artery, directly below the carotid bifurcation. The skin was closed with 5.0 Vicryl sutures (Ethicon). Twenty-eight days after ligation, a plastic 1.7 mm long cast consisting of two rigid halves (Promolding BV, Rijswijk, Netherlands) with a cone-shaped internal lumen ranging from 150 μm (distal) to 300 μm (proximal), was placed around the common carotid artery, directly proximal to the ligation (Fig. 2.1). Before cast placement, presence of flow through the ligated part of the common carotid artery was verified with Doppler-enhanced ultrasound (Vevo 2100, Visualsonics, Toronto, Canada). Four days after cast placement, animals were sacrificed through CO_2 inhalation, exsanguinated by heart puncture and perfused with 4°C PBS. Organs were snap frozen in dry ice-containing ethanol and stored at -80 °C. The left carotid artery was embedded in Optimal Cutting Temperature (OCT) Cryomount compound (Histolab, Gothenburg, Sweden), snap frozen and stored at -80°C.

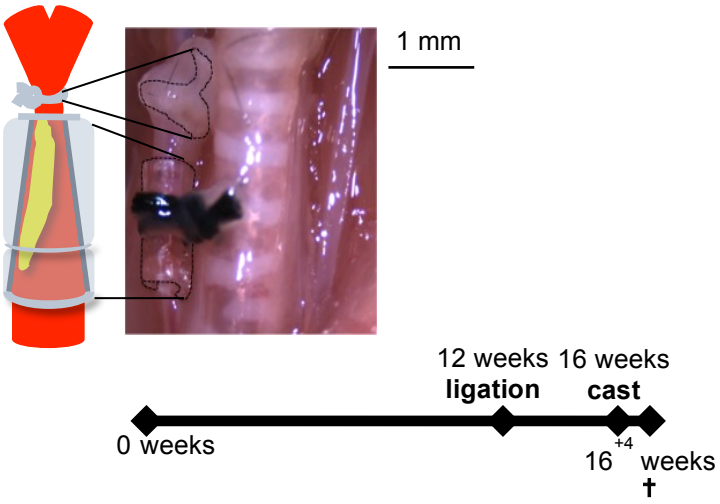

Fig. 2.1 Carotid ligation and cast placement produces plaque formation and rupture in ≈60% of mice.

2.2.5.5 Mediastinal irradiation

For *in vivo* radiation, we used 10-week-old C57BL6/N *Apoe*$^{-/-}$ mice (Taconic, Hudson, NY, USA) weighing 20-30 grams. Anesthesia consisted of intraperitoneal (i.p) injection of 15 µl (50 mg/ml) ketamine (Ketalar, Pfizer, Sollentuna, Sweden), 10 µl (1 mg/ml) of medetomidine (Domitor, Orion Pharma Animal Heath, Sollentuna, Sweden) and 75 µl of phosphate buffered saline (PBS). Depth of anesthesia was verified with a pedal reflex test. Animals were fixated onto a heating pad and covered by a 7 mm thick lead chamber with an open area (1.5 × 2.2 cm) also restricted by a collimator (2.5 × 2 cm) exposing the upper chest and neck region to radiation beams. Mice were placed in a biological irradiator (X-RAD 320, Precision X-Ray, North Branford, USA) and the uncovered area irradiated at 0.7 Gy/min to a total dose of 14 Gy. Control mice received sham irradiation. After irradiation, mice received atipamezole (Antisedan, Orion Pharma Animal Heath, Sollentuna, Sweden) 20 µl (5mg/ml) for anesthesia reversal. During the observation period (3 days, 14 days, or 10 weeks), mice received water and food *ad libitum*. Mice were sacrificed through CO_2 inhalation; plasma and organs were processed as described above.

2.2.5.6 In vivo miRNA modulation

To modulate miRNA expression in mice, animals were injected peritoneally with anti-miRs (LNA-coupled, Exiqon, Vedbaek, Denmark), miRNA mimics (miRVana, Thermo Fisher Scientific), or scrambled control oligonucleotides. Since LNA technology is an effective way to trap and degrade miRNAs in the circulation, no additional vectors are needed. Because miRNA mimics need to be processed by the cell like pri-miRNA transcripts, transfection is more challenging and requires specific chemical packaging. We used Jet-PEI (Polyplus-transfection, Illkirch-Graffenstaden, France) as vector to ensure nuclear delivery. For both groups (Jet-PEI and LNA) we used similarly delivered scrambled control oligonucleotides. LNA and mimic dosage were determined based on the manufacturer's recommendations and varied throughout the experiments.

2.2.6 Cell culture experiments

Throughout all experiments, cells were kept at 37°C in an incubator containing atmospheric oxygen levels and 5% CO_2. For cell detachment, we used 0.05% trypsin-EDTA (Thermo Fisher Scientific). All experiments were repeated three times. For protein analysis, cells were washed with cold (4°C) PBS, lysed with RIPA buffer cocktail (Sigma-Aldrich) containing RIPA buffer, phosphatase- and protease inhibitors, EDTA and sodium azide. The lysate was centrifuged at 14,000 rpm for 15 minutes and protein concentration in the resultant supernatant measured with a bicinchoninic acid assay (Thermo Fisher Scientific). Protein samples were stored at -20°C until analysis. For RNA analysis, cells were washed with warm (37°C) PBS, lysed with QIAzol (QIAgen, Hilden, Germany), and RNA extracted with the QIAgen miRNeasy Mini or Micro Kit (QIAgen) according to the manufacturer's instructions, using the QIAcube RNA preparation robot (QIAgen).

2.2.6.1 Cell types

Primary human carotid artery smooth muscle cells (HCtASMCs), *carotid artery endothelial cells* (HCtAECs) and *aortic smooth muscle cells* (HAoSMCs) were purchased from Cell Applications (San Diego, CA, USA). All three cell types were cultured in complete growth medium (Cell Applications). For *in vitro* experiments, passages 5-9 were used.

HEK293 cells were purchased from Public Health England Culture Collections, Salisbury, UK). Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and 1% PEST.

Mouse aortic smooth muscle cells (MAoSMCs) were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS and 0.2 mg/mL gentamycin (all Gibco).

2.2.6.2 In vitro *miRNA modulation*

Cells were seeded at 125,000 cells/well (6-well plate), 60,000 cells/well (12-well plate), 20,000 cells/well (24-well plate) or 5,000 cells/well (96-well plate). After overnight incubation, growth medium was replaced with Opti-MEM (Gibco, Thermo Fisher Scientific) serum-free medium supplemented with 0.4% FBS (Gibco). Anti-miRNAs, pre-miRNAs, miRNA mimics, or scrambled (negative control) oligonucleotides (all from Ambion, Thermo Fisher Scientific), were combined with Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher Scientific), diluted in Opti-MEM and applied at 75 nM.

2.2.6.3 In vitro *shear stress*

HCtASMCs or HCtAECs were seeded on untreated glass microscopy slides (Flexcell International Corporation, Burlington, VT, USA). ECs were cultured in endothelial cell medium (Cell Applications) and seeded on gelatin-coated slides (Flexcell). The cells were seeded at a density of 750,000 cells per slide (passage 8-9) and were sub-cultured for 48 hours to allow firm cell attachment. Streamer medium consisted of high-glucose DMEM

(Gibco, Thermo Fisher Scientific) supplemented with 1% L-glutamine, 1% PEST and 5% FBS. The shear stress regime included a conditioning phase of 4, 6 and 10 dyn/cm^2 (10 minutes each) followed by 19,5 hours of 12 dyn/cm^2. Total shear stress exposure time was 20 hours. Cells were subjected to either oscillatory or laminar shear stress. Control cells were incubated overnight in the same medium ('static').

2.2.6.4 Luciferase reporter assay

To test miRNA-mediated, posttranscriptional regulation of target genes, a luciferase reporter assay was used. Firefly luciferase gene constructs containing the miRNA sequence predicted to target the mRNA (the 'seed') were purchased from Switchgear Genomics (Active Motif, Carlsbad, CA, USA). As control, two mutated seed sequence constructs were used. In brief, HEK293 cells or HCtASMCs were seeded on 24-well plates (1×10^5 cells/well). At 50-60% confluence, cells were transfected with luciferase reporter plasmid pLS, pLS-target gene-3'UTR or its mutant (100 ng/well, Active Motif), together with control or pre-miRNA (10 nM final concentration) using Dharmafect Duo Transfection Reagent (GE Dharmacon, Lafayette, CO, USA) according to the manufacturer's protocol. After a 24-hour transfection period, luciferase activity was quantified using the LightSwitch Luciferase Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.2.6.5 Radiation

HCtASMCs or HCtAECs were plated and cultured overnight before being placed inside a biological irradiator. Cells received 2 doses of 2 Gy (0.4 Gy/min; total irradiation time 2×5 min) with 24 hours between doses. Control cells were taken out of the incubator, but did not receive irradiation. Cells were harvested 3, 6, or 24 hours after the last radiation exposure, or directly placed in a live-cell imaging incubator (IncuCyte, Essen Bioscience, Hertfordshire, UK) and images taken every 2 hours for the course of 5 days. Proliferation was defined as increase in cell confluence as average of 16 pictures per well in a 6-well plate. For apoptosis measurements, cell medium was supplied with caspase3/7 reagent (Essen Bioscience) at a concentration of 5 μM, as recommended by the manufacturer. Apoptosis was measured as number of positive cells per photographed area.

2.2.7 RNA isolation and qRT-PCR analysis

Cells were washed three times with warm PBS, lysed with 700 μL QIAzol, and scraped off with a cell scraper. Homogenization was achieved by pipetting up and down three times. Tissues were lysed using a tissue homogenizer (ProScientific, Oxford, MS, USA). RNA extraction was performed using the miRNeasy Mini or Micro kit (depending on sample size, QIAgen) following the manufacturer's protocol. For plasma and cell supernatant, we used QIAgen's miRNeasy Mini Kit for plasma RNA extraction with *C. elegans* miR-39 as spike-in control. Total RNA concentration was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). For miRNA analysis, total RNA was diluted in RNAse-free water to a concentration of 2 ng/μL. Five μL (10 ng) was used for miRNA complementary DNA (cDNA) synthesis using the Taqman MicroRNA Reverse Transcription Kit with reverse

transcriptase primers for the selected miRNAs (Supplemental Table 6 and 7, all Thermo Fisher Scientific). For mRNA analysis, we used 200-1,000 ng of total RNA for cDNA synthesis using the Taqman High Capacity cDNA Transcription Kit with random primers. miRNA and mRNA expression was quantified by qPCR using Taqman FAM- or VIC-labeled miRNA and mRNA assays.

2.2.8 miRNA OpenArray

miRNA was purified from 200 µl plasma using the TaqMan miRNA ABC purification kit (Thermo Fisher Scientific, Human panel A and B). ath-miR-159a oligo (synthesized by IDT, 5'UUUGGAUUGAAGGGAGCUCUA3') was added into plasma as spike-in control. miRNA was stored at -80°C until use. The Megaplex primer human pools kit (Thermo Fisher Scientific) was used for miRNA reverse transcription, followed by pre-mplification using Taqman PreAmp Master Mix (Thermo Fisher Scientific). Vendor standard protocols were followed for these procedures.

Samples were then diluted with 0.1×TE buffer, arranged into array order and applied to human miRNA panels using the OpenArray AccuFill system. Taqman OpenArray was performed on a QuantStudio 12K Flex Real-Time PCR system (Thermo Fisher Scientific).

The resulting data was analysed using R software. Quality control included principal component analysis (*prcomp* in R) and analysis of data distribution (*density* in R). Expression of 758 individual miRNAs, including 4 endogenous controls, was tested in all samples. Ct values below 18 and above 38 were set to 'undetermined'. To reduce technical variability, we used global normalization[174]; endogenous controls were removed from the dataset.

2.2.9 Histology

Mouse OCT-embedded frozen tissue was cut into 5 µm thick sections, dried and stored at -80°C. Human vascular material was sampled during surgery, fixed for 48 hours in 2% zinc-paraformaldehyde at room temperature, paraffin-embedded and cut into 5 µm thick sections. Control arteries (carotid, radial, iliac or mammary) were obtained from deceased organ donors without any reported history of cardiovascular disease. Hematoxylin and eosin (HE) staining was used for basic tissue morphology. Lipids were stained with Oil Red O (Sigma-Aldrich). For immunohistochemistry, we used standard biotin-streptavidin-horseradish peroxidase methods.

2.2.10 *In situ* hybridization

For *in situ* hybridization (ISH), we used Exiqon miRCURY LNA double digoxigenin (DIG)-labelled probes with the accompanying kit and protocol (Exiqon). In brief, tissue sections were either de-paraffinized (FFPE) or thawed (frozen) and rehydrated. Nucleases were inactivated with Proteinase K followed by a two-hour hybridization at annealing temperature (30°C below RNA melting temperature). Slides were washed in saline-sodium citrate buffers before hybridized DIG-labelled probes were visualized with subsequent anti-DIG detection methods. Nuclear counterstaining was performed with Nuclear Fast Red (Sigma-Aldrich).

2.2.11 Western blotting

Protein samples (15-30 µg/well) were loaded on Tris-Glycine gradient gels (all Thermo Fisher Scientific). Following electrophoresis and electrotransfer, the PVDF membrane (Bio-Rad, Hercules, CA, USA) was blocked with 5% milk in Tris-buffered saline with 0.01% Tween (TBST) or 5% BSA-TBST (Bio-Rad) before incubation with primary antibody overnight at 4°C. After secondary antibody incubation, we used standard chemiluminescence detection methods and ImageJ analysis software to determine and quantify protein expression.

2.2.12 Statistical analysis

SPSS Statistics version 22 (IBM) was used to analyze patient data. To compare two groups, a Student's *t* test was used. Paired data were analyzed by paired-samples *t* test. Differences between ≥2 groups versus a control group were analyzed with One-way ANOVA plus Bonferroni correction for multiple comparisons. Percentages and proportions were analyzed with Chi-square testing. Mann-Whitney U test was used to compare two groups when data were considered non-parametric. Statistical analysis for experiment data was carried out using R software, or Graphpad Prism software version 6.0b or higher. Differences in RNA expression were calculated as fold change versus control using the mean delta Ct (ΔCt, defined as $Ct^{\text{target RNA}}$ minus $Ct^{\text{endogenous control}}$ or $Ct^{\text{global mean}}$) within groups.

In Project II, we used PASS software (Department of Biostatistics, College of Public Health, UNMC, Omaha, NE) to calculate the sample size needed to achieve 96% power for each miRNA, to detect a true difference in expression of at least 0.1 with estimated group standard deviations of 0.1 and with an experiment-wise error rate of 0.0500 using a two-sided two-sample *t* test. We found a required sample size of 200 cases (patients with an abdominal aortic diameter (AAD) of >3 cm at baseline) and 150 controls (AAD <3 cm). Differences in RNA expression were calculated as fold change versus control using the mean ΔCt (defined as $Ct^{\text{target RNA}}$-$Ct^{\text{endogenous control}}$ or $Ct^{\text{global mean}}$) within groups. The miRNA OpenArray results were analysed with One-Way ANOVA (control versus stable AAA versus AAA_{rupt}), and a Welch's *t* test (AAA versus control). ANOVA was done with the R stats function *aov* (linear fitting) for each miRNA. Tukey's *post hoc* test was performed on miRNAs with a significant ANOVA p-value after Bonferroni correction. ANOVA was executed as *aov*(Ct_norm~Group) where Group was a factor of the type of AAA (control/stable AAA/AAA_{rupt}).

2.3 RESULTS

The following section includes a summary of results from the manuscripts constituting the thesis. For detailed results, please refer to the relevant paper.

2.3.1 miR-24 in AAA (I)

2.3.1.1 miRNAs in mouse AAA

We compared miRNAs and mRNAs expressed in aneurysmatic abdominal aortic tissue from mice receiving porcine pancreatic elastase (PPE) infusion with those in tissue from sham-treated (saline-infused) mice. Of all the downregulated individual miRNAs, miR-24 had the most significant negative correlation with upregulated genes and *in situ* hybridization (ISH) showed diminished miR-24 expression throughout the aneurysmal aortic wall of PPE mice (Fig. 2.2A). Chitinase 3-like 1 (*Chi3l1*), a pro-inflammatory 18-glycosyl hydrolase produced and secreted by differentiated macrophages[175], was the only miR-24 target gene substantially altered at day 7, 14, and 28 after PPE induction (Fig. 2.2B).

In another AAA model of systemic angiotensin II (ANGII) infusion into *Apoe*$^{-/-}$ mice over a time course of 28 days, miR-24 was again significantly downregulated at all three time points (days 7, 14 and 28) during aneurysm development (Fig. 2.2C), and *Chi3l1* expression correlated negatively with miR-24 expression (Fig. 3.1.2c). ISH for miR-24 and immunohistochemistry (IHC) using a mouse macrophage antibody (F4/80) revealed that miR-24 co-localized with activated macrophages in aortic aneurysmal mouse tissue (post-PPE day 7; Fig. 2.2E). Double immunofluorescence of the vascular smooth muscle cell (SMC) marker alpha actin (α-SMA) and F4/80 with CHI3L1 indicated co-localization (orange in merged images) of CHI3L1 with SMCs as well as macrophages. *In vitro* experiments with peritoneal macrophages harvested from ANGII-AAA mice as well as RAW 264.7 macrophages showed a decrease in miR-24, affecting *Chi3l1*, dependent on IL6 stimulation, confirming a negative correlation between inflammation and miR-24 expression.

2.3.1.2 Regulation and modulation of miR-24 in vitro and in vivo

To elucidate the mechanistic effects of miR-24 modulation in AAA, we transfected macrophages and SMCs *in vitro* with anti-miR-24 or pre-miR-24 under various inflammatory stimuli. These experiments confirmed that pro-inflammatory CHI3L1 expression was miR-24 dependent. In both *in vivo* models of AAA development, pre- and anti-miR-24 delivery regulated Chi3l1 expression and, more importantly, prevented respectively augmented AAA expansion and rupture (Fig. 2.2F).

2.3.1.3 miR-24 in human AAA

The clinical relevance of miR-24 as a biomarker for AAA was assessed using human infrarenal aortic tissue from patients undergoing open AAA repair surgery versus non-dilated organ donor controls. As in our animal models, miR-24 was significantly downregulated and *CHI3L1* upregulated in human AAA tissue. miR-24 levels were not different for small or large AAAs, but tissue expression of *CHI3L1* correlated positively with disease severity (Fig. 2.2F). In a similar

manner, plasma miR-24 expression was decreased in patients with AAA as compared to age-medication- and risk factor-matched controls. Patients with peripheral vascular occlusive disease did not express these changes in miR-24. Again, plasma miR-24 could not distinguish patients with small from those with large aneurysms, whereas *CHI3L1* levels could (Fig. 2.2G).

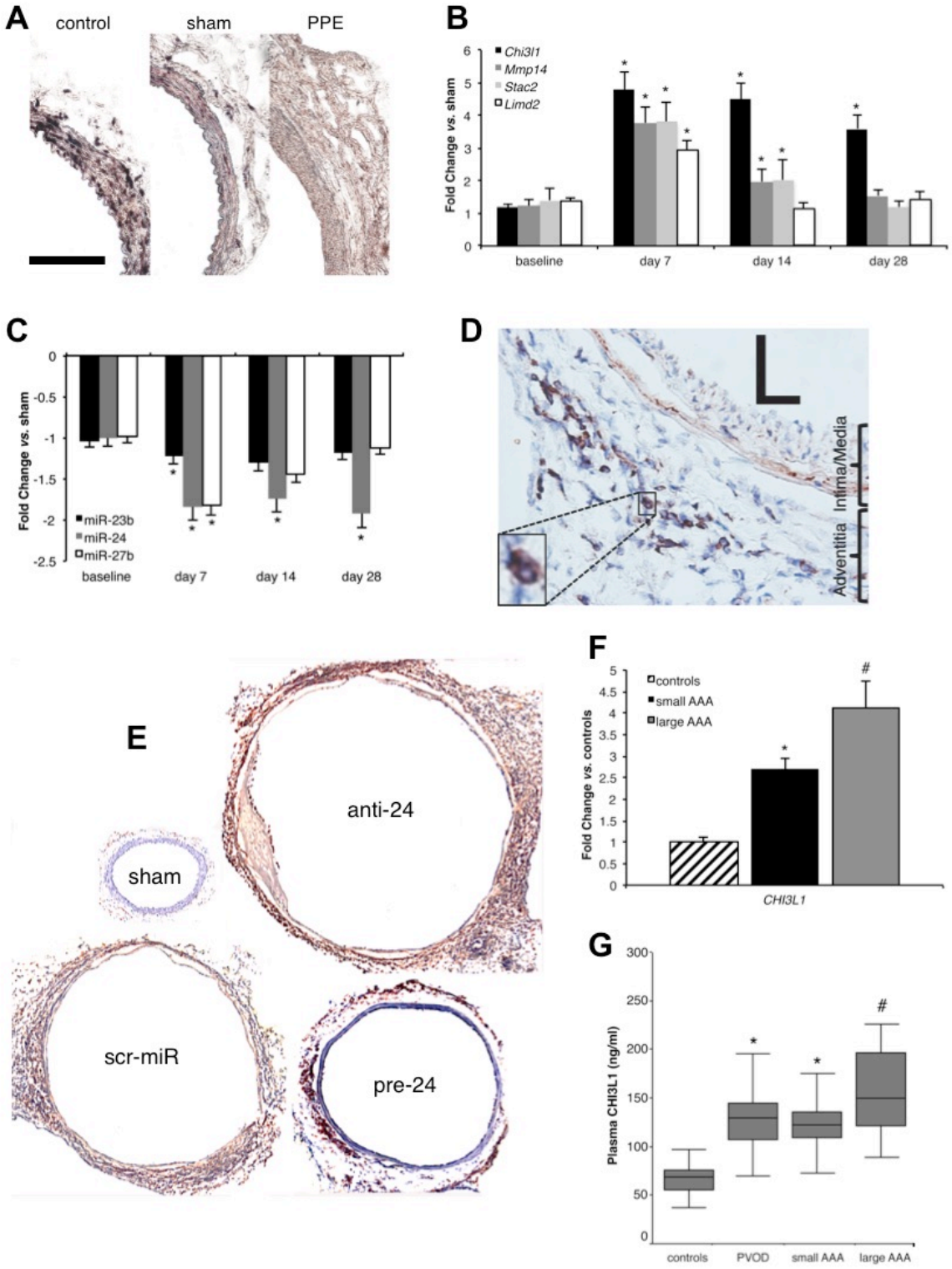

Figure 2.1 miR-24 prevents experimental AAA growth through inhibition of chitinase 3-like 1. (**A**) *In situ* hybridization for miR-24 in control aorta, sham and PPE 14 days after AAA induction. Bar, 400 μm. (**B**) miR-24 target gene expression at day 7 after PPE surgery. (**C**) miR-23b-24-27b cluster expression in aortic tissue from mice treated with AngII compared to NaCl-infused mice. (**D**) In aortic macrophages from PPE-treated mice, miR-24 co-stained with CHI3L1. (**E**) Different-sized aneurysms in PPE mice treated with miR-24 modulators. (**F**) In human aortic tissue, *CHI3L1* expression increased with AAA size. (**G**) In plasma, CHI3L1 protein could distinguish patients with large AAA from patients with small AAA, PVOD and healthy controls. *p<0.05 versus sham/controls. #p<0.05 versus controls/small AAA.

2.3.2 microRNAs as AAA biomarker (II)

2.3.2.1 Plasma miR-99b-3p, -99b-5p, -141-3p, and -643 expression predicts AAA_{rupt} or expansion

Expression of 754 miRNAs was assessed in 195 AAA patients versus 184 matched controls from the Dutch Second Manifestations of Arterial disease (SMART) cohort[161]. After Bonferroni correction for multiple comparisons, miR-99b-3p, miR-99b-5p, miR-141-3p, and miR-643 were significantly deregulated in plasma from patients with AAA_{rupt} (rupture or expansion requiring surgery) as compared to controls and those with stable AAA. miR-99b-3p and miR-643 were downregulated; miR-99b-5p and miR-141-3p were upregulated (Fig. 2.2A). There were no significantly different miRNAs in plasma from patients with AAA at baseline versus controls.

2.3.2.2 miR-99b is deregulated in murine models of AAA

In aortic tissue from mice subjected to PPE or angiotensin II infusion, miR-99b (-5p and -3p) were consequently downregulated at 7 and 14 days after treatment induction, making miR-99b-3p the only miRNA in mouse tissue regulated in the same direction as in human plasma.

2.3.2.3 miR-99b expression is altered in ruptured AAA

In tissue explanted during surgery due to ruptured AAA, as compared to tissue from non-ruptured controls, miR-99b expression was decreased (Fig. 2.2B).

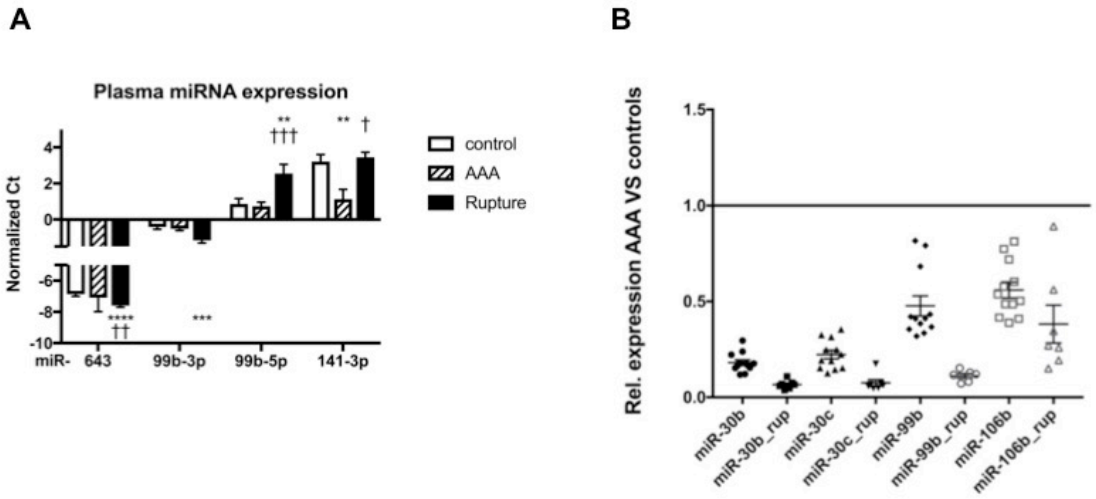

Figure 2.2. Plasma and tissue expression of miR-99b-3p was altered in AAA and AAA_{rupt}. (**A**), miRNA expression in plasma from AAA and AAA_{rupt} patients versus controls. **p<0.01; ***p<0.001; ****p<0.0001 versus control. †p<0.05; ††p<0.01; †††p<0.001 versus AAA. Significance calculated in One-Way ANOVA; p values adjusted with Tukey's *post hoc* test after Bonferroni correction. (**B**), Relative expression of miR-30b, -30c, -99b-3p, and -106b in tissue from AAA patients (ruptured and non-ruptured) versus control tissue.

2.3.3 miR-210 in atherosclerotic plaque stability (III)

2.3.3.1 Human carotid artery miRNA and target profiling

In plasma from BiKE, sampled at the carotid lesion site after clamping of the carotid artery, microarray analysis showed that out of 742 tested transcripts, miR-210 was the most substantially (1.5 fold) and significantly downregulated miRNA in 7 symptomatic versus 5 asymptomatic patients. In peripherally sampled plasma, miR-210 was undetectable in the majority of samples, and there was no difference in expression levels between symptomatic and asymptomatic patients.

To find a more accurate localization in order to define a potential role for miR-210 in plaques, we performed *in situ* hybridization for miR-210 in histologically graded ruptured versus stable plaque, using a grading method issued by the Atherosclerosis Council of the American Heart Association[20]. miR-210 was expressed in the intima-medial layer of healthy vessels and stable plaques, but not in that of ruptured lesions (Fig. 2.3A). Laser capture microdissection of fibrous cap tissue confirmed suppression of miR-210 in ruptured versus stable fibrous caps (Fig. 2.3B).

A potential functional role for miR-210 regulation in fibrous caps was explored by examining deregulation of miR-210 target genes in previously published BiKE microarray datasets[163]. The well-known tumor suppressor gene *APC* caught our attention, having an important inhibitory function in canonical Wnt signaling, which in SMCs contributes to the balance between proliferation and apoptosis[176,177]

2.3.3.2 miR-210 and APC are deregulated in murine models of carotid injury

To gain mechanistic insight into the function of miR-210 in the vessel wall, we aspired to create a more controlled vascular injury setting in which we could measure miR-210 and its targets. To achieve this, we used two different murine models of carotid manipulation. First, we saw a decrease in miR-210 in rat carotid arteries injured with an endovascular balloon, as compared to the intact contralateral carotid (Fig. 2.3C). Concordant with miR-210 downregulation, *Apc* mRNA expression was increased in the critical two-week post-injury period of remodeling and neointima formation.

We created a more advanced atherosclerosis setting, ligating and subsequently placing a cast around the right carotid artery of male *Apoe*$^{-/-}$ mice. This resulted in plaque formation in 100%, and subsequent plaque rupture in 58% of animals. *In situ* hybridization of ruptured versus stable carotid plaques showed a decrease in miR-210 expression specifically in the fibrous cap (Fig. 2.3D), while whole vessel miR-210 expression, as measured with qRT-PCR, was not different.

2.3.3.3 miR-210 mimics prevent murine carotid plaque rupture

Having established an association between miR-210 and APC expression on one side, and plaque vulnerability on the other, we used the mouse plaque rupture model to investigate whether miR-210 modulation can influence plaque stability and rupture rate. First, we confirmed that we could successfully deliver (scrambled) fluorescently labeled oligonucleotides, via intraperitoneal injection, in the vessel wall of treated carotid arteries (Fig. 2.3E). miR-210 inhibition using LNA-

coupled anti-miR-210 did not significantly increase plaque rupture compared to scrambled control, but miR-210 mimics substantially decreased the rupture rate (Fig. 2.3F).

In a series of experiments to assess RNA- and protein marker expression, we could show that miR-210 mimic therapy resulted in decreased APC in mouse carotids, increased SMC content, and prevention of SMC apoptosis. Treatment with miR-210 mimics did not change other atherosclerosis parameters such as cholesterol or triglyceride levels, or atherosclerosis development *per se* as assessed by aortic arch *en face* Sudan IV- and aortic root Oil Red O staining.

2.3.3.4 miR-210 modulation affects APC expression and HCtASMC apoptosis in vitro

Based on previous knowledge about endothelial cell layer insufficiency[16] and the influence of fluid shear stress on fibrous cap (in)stability[178], we asked whether exposure of human carotid artery smooth muscle cells (HCtASMCs) *in vitro* to physiologic (laminar) or pathologic (oscillatory) fluidic flow would alter miR-210 and APC expression. When exposed to 12 dyn/cm^2 of oscillatory flow, HCtASMCs showed loss of miR-210 expression and upregulation of *APC* (Fig. 2.3G).

In subsequent *in vitro* experiments, we could confirm our *in vivo* findings that miR-210 mimics inhibit APC protein expression and apoptosis. In addition, we explored the downstream effects of APC modulation on Wingless-related integration site (Wnt) signaling in HEK293 cells and HCtASMCs. In canonical Wnt signaling, APC is part of a destruction complex preventing β-catenin from entering the nucleus, a translocation necessary to co-activate proliferative transcription factors including the T-cell factor/lymphoid enhancer factor (TCF/LEF) family[179]. In HEK293 cells as well as HCtASMCs, miR-210 mimics stimulated TCF/LEF signaling, an effect that was abolished by *APC* overexpression. Taken together, our *in vitro* findings point towards miR-210 as a crucial regulator of advanced atherosclerotic lesion fate by stimulating canonical Wnt signaling via inhibition of APC in SMCs (Fig. 2.3H).

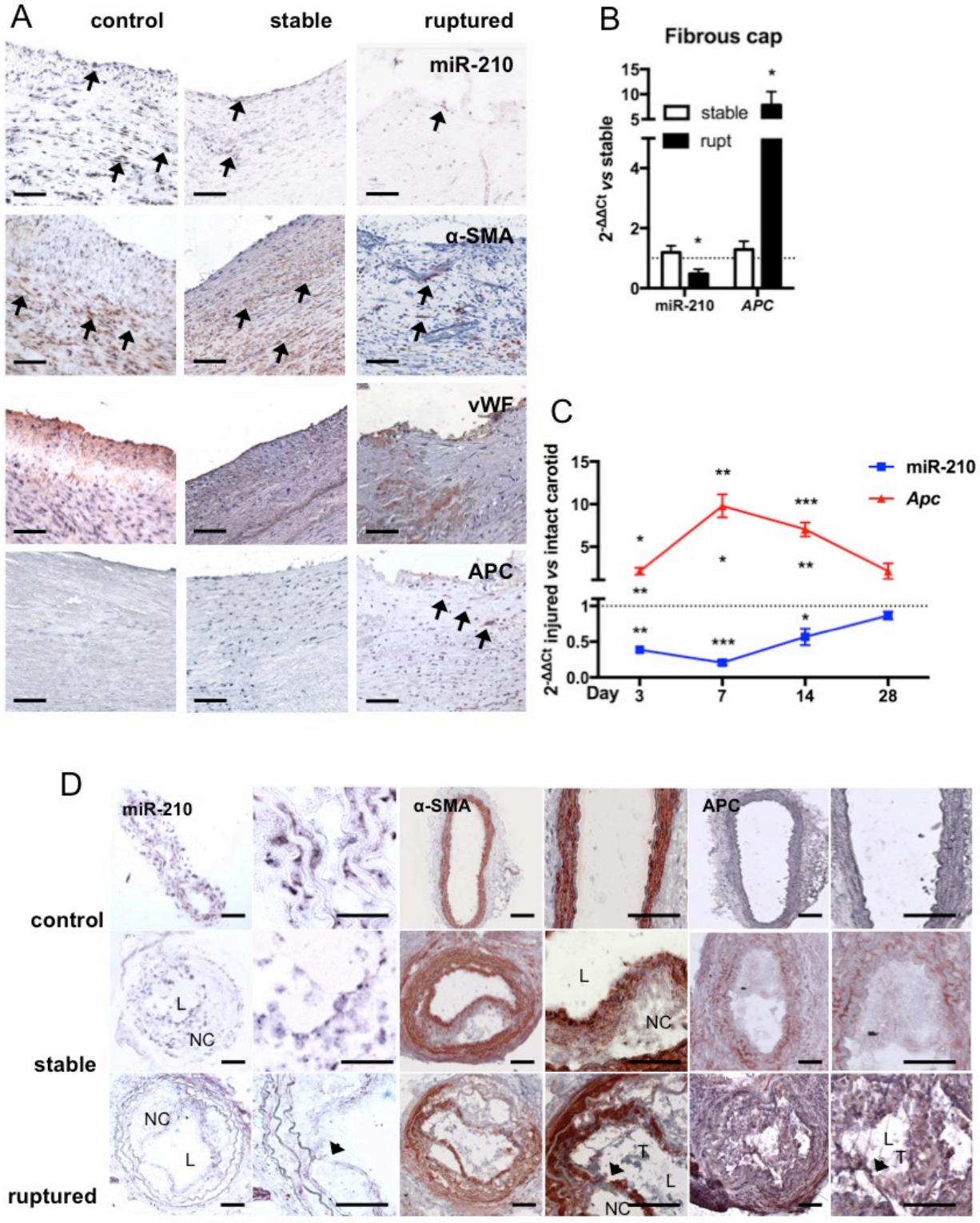

Figure 2.3. miR-210 in atherosclerotic plaque stability. A, miR-210 expression associated with carotid smooth muscle as well as endothelial cells. APC co-localized with smooth muscle cells in ruptured, but not stable, plaques. **B**, LCM of carotid fibrous caps showed significantly decreased miR-210 and increased *APC* mRNA in ruptured versus stable plaques. Mean+SEM. *p<0.05 in Student's *t* test, *n*=10 per group. **C**, During the first two weeks after balloon injury, rat carotid tissue showed inverse regulation of miR-210 and *Apc* mRNA. Mean+SEM. ***p<0.001; **p<0.01; *p<0.05 in paired-samples *t* test. **D**, As in human lesions, miR-210 localized towards the fibrous cap, and is associated with SMCs. Bars, 100 μm.

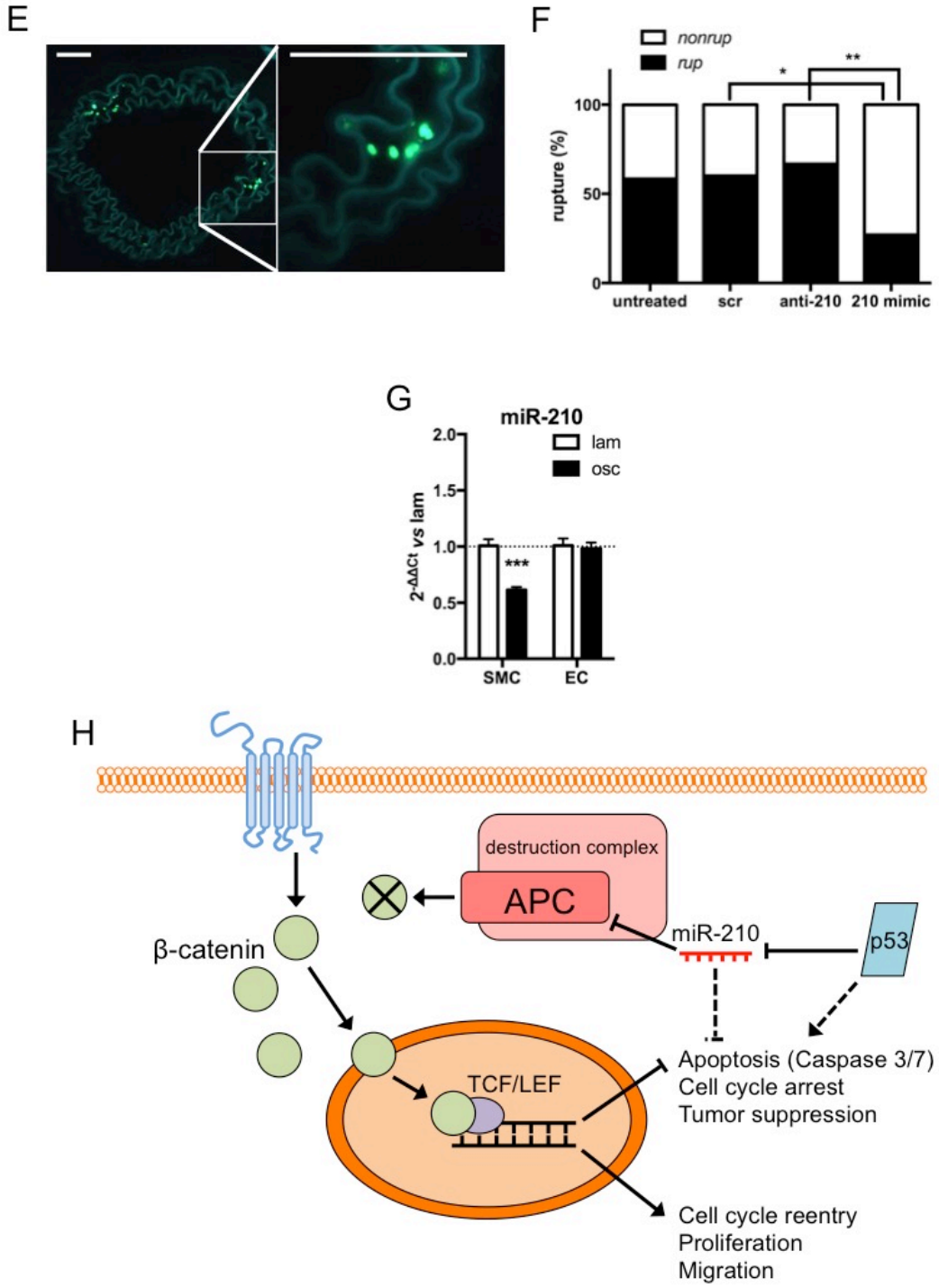

Figure 2.3 continued. E, FAM-labeled miRNA modulators were detected in the *tunica media* of carotid arteries where plaque rupture was induced. Bars, 100 μm. **F**, Intravenous administration of miR-210 mimics significantly reduced mouse carotid plaque rupture compared to scrambled miRNA and anti-miR-210 plaque rupture rate. **p<0.01, *p<0.05 in Chi^2 test. **G**, Oscillatory (*osc*, pathologic) shear stress resulted in downregulation of miR-210 in human carotid artery smooth muscle cells (SMC) as compared to laminar (*lam*, physiologic) shear stress. miR-210 expression remained unchanged in response to shear stress alteration in human carotid artery endothelial cells (EC). Mean+SEM. *p<0.05 in One-way ANOVA versus static control. **H**, Descriptive cartoon of the proposed mechanism of miR-210 dependent inhibition of APC-mediated apoptosis.

2.3.4 miR-29b and miR-146b in radiation-induced vascular inflammation (IV)

2.3.4.1 miR-29b and miR-146b, and targets linked to inflammation and wound repair, are deregulated in radiated versus non-radiated vascular tissue

In 15 pairs of arterial tissue samples from 15 patients undergoing microvascular free tissue transfer reconstructions after radiation for tumors in the head and neck area, we compared the expression of 11 well-known miRNAs in cardiovascular disease, cancer, or both. miR-29b and miR-146b were significantly deregulated in irradiated tissue (Fig. 2.4A). *In situ* hybridization of miR-29b and miR-146b in these arteries corresponded with the whole-vessel expression of both miRNAs. miR-29b and miR-146b could be found throughout the vessel wall, but with a clear predilection for the medial layer (Fig. 2.4B).

Using previously published RNA analyses of these samples, we searched for experimentally validated miR-29b and miR-146b gene targets regulated in a direction inverse to that of the miRNA. In radiated versus non-radiated tissue, pentraxin 3 (PTX3) was the most profoundly upregulated miR-29b target. Dipeptidyl-peptidase 4 (DPP4) was also significantly upregulated. In immunohistochemically stained formalin-fixed paraffin-embedded arterial tissue from the same patients, we could localize PTX3 and DPP4 protein expression in the intimal and medial layer of radiated arteries, whereas expression in their non-radiated counterparts was markedly lower (Fig. 2.4B).

2.3.4.2 Radiation affects vascular miR-29b and miR-146b expression in a murine model

We subjected male *Apoe*$^{-/-}$ mice to radiation using a model first described by Stewart *et al*[180]. In mice receiving a single radiation dose of 14 Gy in a designated mediastinal area including the heart and large vessels, thoracic aortic- and carotid tissue showed significant downregulation of miR-29b after three days (carotid) and 14 days (thoracic aorta) (Fig. 2.4C-D). miR-146b showed an upward, albeit non-significant, trend.

2.3.4.3 In SMCs, miR-29b reacts to radiation in vitro*, and its modulation alters the cellular response to radiation*

When exposed to 2×2 Gy of radiation, miR-29b expression in SMCs decreased (Fig. 2.4E). Treating these cells with either miR-29b mimics or anti-miR-29b, we could change the gene expression of *PTX3* (Fig. 2.4F). *DPP4* gene expression was not directly affected but DPP4 protein expression was – this is a common phenomenon in miRNA research where an mRNA is rendered inactive by the miRNA but still detectable with PCR.

2.3.4.4 miR-29b modulation changes DPP4 and PTX3 expression in mouse vascular tissue

In radiated mice, we found that treatment with miR-29b mimics decreased DPP4 and PTX3 protein expression in the atherosclerotic plaque (DPP4) and vessel wall (PTX3) (Fig. 2.4F).

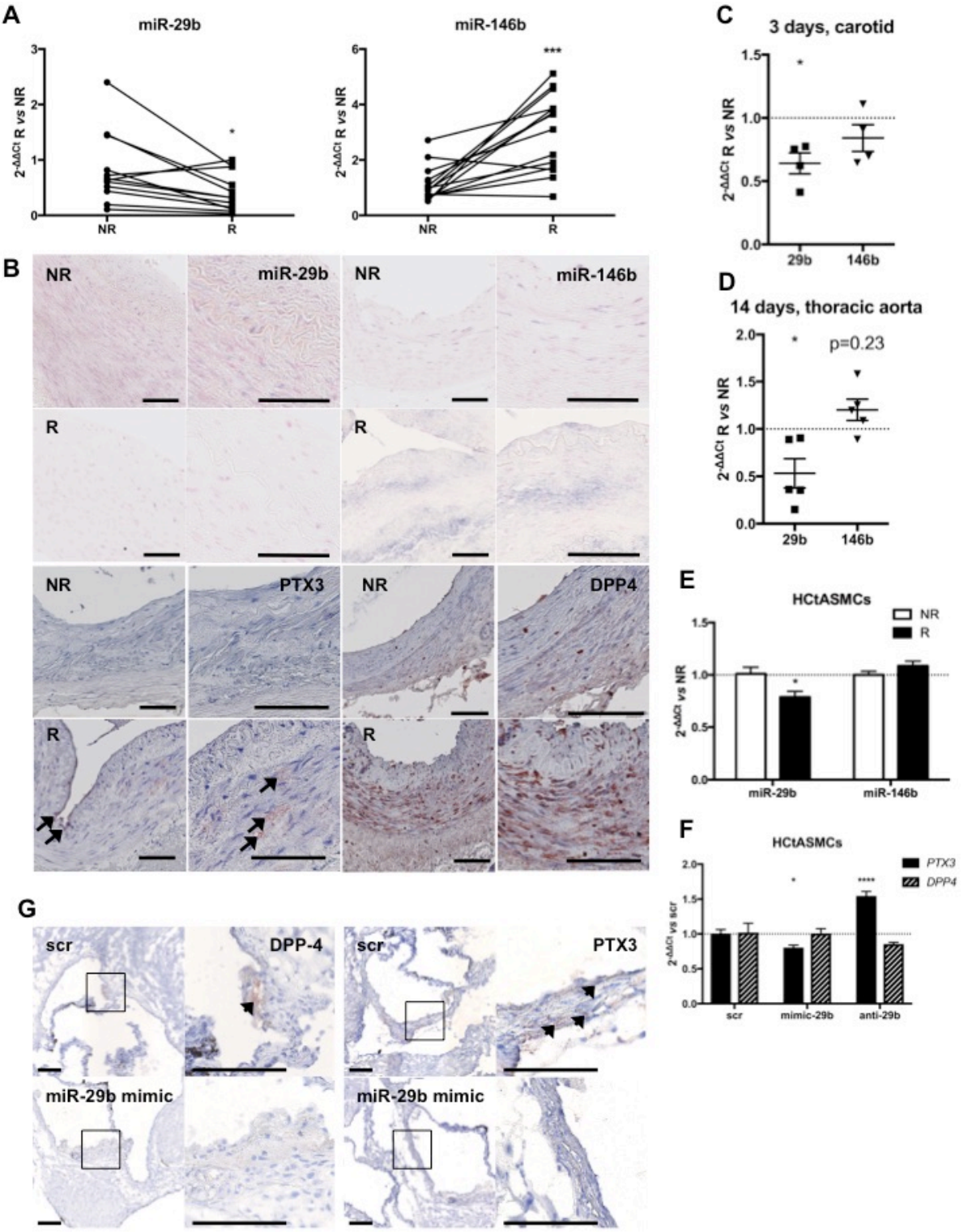

Figure 2.4. Irradiation affects arterial miR-29b and miR-146b expression in human arteries. A, miR-29b is downregulated, miR-146b upregulated after irradiation. *n*=15 in each group. *p<0.05: ***p<0.001 in paired-samples *t* test. **B**, both miRNAs localize towards the *tunica media*. **C** and **D**, miR-29b is significantly deregulated in irradiated mouse tissues versus tissue from sham-irradiated mice. Mean±SEM. **E**, human carotid artery SMCs (HCtASMCs) downregulate miR-29b after irradiation. **F**, HCtASMC *PTX3* mRNA expression can be changed with miR-29b modulators. Mean+SEM; *p<0.01; ****p<0.0001 in Student's *t* test. **G**, DPP4 and PTX3 protein expression in scrambled control- (scr) versus miR-29b mimic treated mice. Bars, 100 μm.

3 DISCUSSION

Among the pharmacological approaches to battle atherosclerotic disease, traditional agents such as anti-hypertensives, statins, and platelet aggregation inhibitors have greatly improved patients' clinical perspectives. Although these currently applied pharmaceuticals can alleviate symptoms of cardiovascular disease, they mainly interfere with specific early triggers of vascular remodeling (e.g. RAAS activation). As cardiovascular disease ought to be seen as a combined sequela of different inducing events (mechanical triggers, RAAS activation and inflammation), these strategies appear only partially effective. The incessant advance of cardiovascular disease worldwide, in the Western world especially due to ageing populations, demands therapies that more directly target the structural changes and alterations of vascular signaling underlying the process of remodeling, not only in early stages, but also in advanced disease. Furthermore, atherosclerosis leading to cardiovascular events needs to be better distinguished from vascular remodeling associated with normal ageing. The biological robustness miRNAs can add to a patient's risk profile can help cardiovascular medicine to live up to the expectations of 'personalized medicine'.

The discovery of ncRNAs as critical contributors to gene expression and genome maintenance has initiated a surge in ncRNA research. As human genome banks expand, increasingly detailed information about the diversity of the RNA landscape becomes available, and in the present-day society these discoveries are readily presented as future groundbreaking medical advances. Indeed, it is increasingly difficult to find a biological process not influenced by ncRNAs; ncRNA therapeutics can target and modify cellular messages previously considered as 'un-druggable', and this understandably has sparked wide medical and economic interest in ncRNA modifying compounds. Yet, thus far, the modest number of clinical ncRNA successes does not reflect the auspiciousness of perspectives depicted in preclinical and *in vitro* studies. In our view, inability of the ncRNA field to live up to its promises is partly due to a lack of mechanistic translational studies, which combine patient data with *in vitro* and *in vivo* findings. Even more importantly, potential therapeutic ncRNA modifiers identified by translational studies need to be applied *in vivo* in a manner relevant to the targeted human pathology, for example locally, and by using safe as well as effective carriers.

3.1 THE CURRENT PLACE OF NCRNAS IN CARDIOVASCULAR MEDICINE

As biomarkers, miRNAs are at the forefront of the ncRNA domain, because their relative stability and co-expression patterns can provide a detailed signature of a disease. miRNAs can help to classify diseases, predict drug efficacy, and improve risk prediction. We showed that miR-24 and miR-99b levels in plasma can indicate AAA progression by reflecting the cellular state and behavior of aortic macrophages and SMCs. Use of these expression data adds vascular biological robustness to ultrasound measurements. This can provide a better prediction of AAA rupture and is therefore a step towards better allocation of AAA correction surgery.

The discordance between studies, the absence of proper normalizing controls, and the multitude of confounding factors such as medication, inflammation, and diet, are major remaining challenges in the candidacy of miRNAs as biomarkers[85]. For the field to overcome these issues, development of large-scale studies and standardization of detection methods are essential. In all studies, human material was the starting point for ncRNA expression analysis. Potential mechanisms behind the pathology were then tested *in silico*, *in vitro* and *in vivo*.

3.2 MIR-24, MIR-210 AND MIR-29B IN SELECTED CARDIOVASCULAR DISEASES

As therapeutic agents, ncRNAs can have potent effects on disease phenotypes *in vitro* and *in vivo*. First, we could show in mouse models of AAA as well as in human aortic SMCs that miR-24 has an essential role in macrophage inflammatory activity through inhibition of CHI3L1 (Paper I). In mice, interaction between miR-24 and *Chi3l1* controlled inflammatory activity within the dilated aortic wall. Through enhanced blockage of *Chi3l1* with pre-miR-24, inflammatory activity was dampened, resulting in limited AAA expansion. *In vitro* experiments showed that NF-κB is partly responsible for miR-24 downregulation in SMCs exposed to inflammatory activity. In macrophages and SMCs, miR-24 downregulation stimulated cytokine synthesis and adhesion molecule expression, suggesting a locally acting vicious circle of inflammation, miR-24 downregulation, and AAA expansion. This local element in AAA pathology makes miR-24 and CHI3L1 interesting potential targets for drug eluting balloon- or stent-mediated expansion prevention.

In Paper III, we found miR-210 to be strongly and significantly downregulated in and around unstable fibrous caps of carotid atherosclerotic plaques. In three different *in vivo* models of vascular injury, we confirmed the association of miR-210 with arterial remodeling and unstable plaque. Using systemically injected miR-210 mimics, we successfully inhibited apoptosis of SMCs, the source of fibrous cap-associated miR-210, by blocking the tumor suppressor gene APC in these cells. We showed *in vitro* that SMCs can be rescued from apoptosis through miR-210 overexpression causing enhanced posttranscriptional inhibition of APC. APC inhibition leads to stimulation of canonical Wnt signaling, which in SMCs can direct a variety of atherosclerotic processes, e.g. proliferation, cell migration, differentiation and apoptosis, leading to a stable plaque phenotype. A point of concern regarding miR-210 is the reported link between high miR-210 expression with various forms of cancer[181]. Our findings identifying miR-210 as inhibitor of one of the most infamous cancer genes, add to this evidence. To prevent potentially tumorigenic off-target effects, plaque-stabilizing miR-210 therapy should ideally be applied locally with stents or balloons.

In radiated SMCs, we inhibited PTX3 production and collagen synthesis by increasing the availability of miR-29b, a miRNA able to target PTX3 and thereby development of fibrosis (Paper IV). PTX3 is a direct actor in innate inflammation, and its inhibition with the use of miR-29b mimics could be a potent therapeutic approach to thwart radiation-induced vasculopathy.

The fact that some miRNAs are tissue specific has advantages for their diagnostic and therapeutic application, but only a minority of miRNAs shows tissue specificity[182]. We and others have demonstrated that systemic delivery of miRNA mimics or anti-miRs presents a considerable load of these modulators to the circulation, where they might interact with serum proteins, accumulate in organs such as the kidney, liver and lungs, and possibly also exert adverse off-target effects at these sites[102,183]. Local application, allowing lower dosages and preventing distant off-target effects, increases safety as well as efficiency. Developing new targeted delivery methods with the use of nanoparticles, conjugates, targeting ligands *et cetera* has become a rapidly expanding field of research, where innovative and creative designs are met with great academic as well as financial interest.

The inhibitory effect of a miRNA on one mRNA is often modest, but this fact is compensated by the multiplicity of mRNA targets one miRNA can have. Examples are miR-29, which has multiple targets in the fibrotic response[110], and miR-145, which regulates components of actin cytoskeletal function[184]. This pleiotropic character, although biologically efficient, complicates miRNA therapy strategies and further stresses the importance of local distribution of miRNA modifiers if they are to be applied as treatment.

3.3 MIR-99B AS BIOMARKER FOR AAA

In Paper II, we showed that plasma miR-99b expression can, in a population at risk for vascular disease, distinguish between individuals with normal caliber aortas, stable AAA and rupture-prone aneurysms. In addition, miR-99b deregulation was observed in aortic tissue from patients with ruptured AAA. That the expression of this miRNA is also distorted by AAA induction in mice, suggests a role for miR-99b in the inflammatory process underlying AAA. Downregulation of miR-99b might be involved in extracellular matrix breakdown, when the inhibitory actions of this miRNA on tumor growth factor-beta (TGF-β)-induced epithelial to mesenchymal transition are thwarted.

3.4 FUTURE PERSPECTIVES

The number, quality and reproducibility of translational studies will ultimately determine the success of ncRNA therapeutics for human disease. Application of ncRNAs, identified *in silico* and *in vitro*, in *in vivo* disease models is an essential step in translation. This research strategy, when applied, will undoubtedly also unveil the challenges in developing ncRNA therapeutics: delivery method, pleiotropy, and stability.

For cardiovascular disease, coated stents or balloons lend themselves readily for miRNA delivery[102,185], and this possibility should be explored in large animal models, if not clinically. Ultrasound-triggered microbubbles face a longer path towards clinical application, but their non-invasive properties are attractive features in the vulnerable cardiovascular patient group[186].

The future for clinical application of ncRNAs lies in the discovery of more in-depth knowledge of the different ncRNAs and their function, better detection methods and higher

efficacy and specificity of ncRNA therapeutics. Another important task for the ncRNA field is to draw attention to the message that ncRNAs, like proteins, are essential functional cell elements, and that in cellular biology, cooperation between RNA and proteins is a rule rather than an exception. Only this perspective, which fundamentally differs from the unidirectional 'central dogma of molecular biology', does justice to the pivotal role of ncRNAs in health and disease.

4 CONCLUSION

In this thesis, we used human material to detect ncRNAs marking and regulating the discourse of three cardiovascular diseases. *In vivo*, we found that modulation of selected ncRNAs can be used to direct cardiovascular pathologic processes towards a protective, disease-limiting phenotype. Reducing the vascular pathology *in vitro* to biological processes involving different human cell types such as SMCs, ECs and macrophages, we found miRNA-mRNA interactions that explain the behavior of vascular cells, not only in these specific diseases, but in atherosclerosis in general. With this work, we have contributed evidence to the notion that ncRNAs are essential cellular elements, equaling proteins in importance and potency. Given the proven clinical potential of siRNA-based ncRNA-modifying techniques, and in line with the recent surge in siRNA delivery techniques, ncRNA therapies for cardiovascular disease are becoming near-future therapeutic options, rather than distant prospects.

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6 REFERENCES

1. Schlatmann TJ, Becker AE. Histologic changes in the normal aging aorta: implications for dissecting aortic aneurysm. Am J Cardiol 1977;39:13-20.

2. Wang JC, Bennett M. Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. Circ Res 2012;111:245-59.

3. Juonala M, Viikari JS, Kahonen M, et al. Life-time risk factors and progression of carotid atherosclerosis in young adults: the Cardiovascular Risk in Young Finns study. European heart journal 2010;31:1745-51.

4. Tuzcu EM, Kapadia SR, Tutar E, et al. High prevalence of coronary atherosclerosis in asymptomatic teenagers and young adults: evidence from intravascular ultrasound. Circulation 2001;103:2705-10.

5. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature 2011;473:317-25.

6. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012;380:2095-128.

7. Yusuf S, Rangarajan S, Teo K, et al. Cardiovascular risk and events in 17 low-, middle-, and high-income countries. N Engl J Med 2014;371:818-27.

8. Mendis S, Davis S, Norrving B. Organizational update: the world health organization global status report on noncommunicable diseases 2014; one more landmark step in the combat against stroke and vascular disease. Stroke 2015;46:e121-2.

9. Law MR, Wald NJ, Rudnicka AR. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. BMJ (Clinical research ed) 2003;326:1423.

10. van Vark LC, Bertrand M, Akkerhuis KM, et al. Angiotensin-converting enzyme inhibitors reduce mortality in hypertension: a meta-analysis of randomized clinical trials of renin-angiotensin-aldosterone system inhibitors involving 158,998 patients. European heart journal 2012;33:2088-97.

11. Welch HG, Albertsen PC, Nease RF, Bubolz TA, Wasson JH. Estimating treatment benefits for the elderly: the effect of competing risks. Ann Intern Med 1996;124:577-84.

12. Fauci ASB, E.; Kasper, D. L.; Hauser, S. L.; Longo, D. L.; Larry Jameson, J.; Loscalzo, J. Harrison's Principles of Internal Medicine. 17 ed2008.

13. Pillarisetti S. Lipoprotein modulation of subendothelial heparan sulfate proteoglycans (perlecan) and atherogenicity. Trends in cardiovascular medicine 2000;10:60-5.

14. Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. Circulation 2007;116:1832-44.

15. Brown MS, Goldstein JL, Krieger M, Ho YK, Anderson RG. Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. The Journal of cell biology 1979;82:597-613.

16. Shi ZD, Tarbell JM. Fluid flow mechanotransduction in vascular smooth muscle cells and fibroblasts. Ann Biomed Eng 2011;39:1608-19.

17. Ketelhuth DF, Hansson GK. Adaptive Response of T and B Cells in Atherosclerosis. Circulation research 2016;118:668-78.

18. Tabas I, Garcia-Cardena G, Owens GK. Recent insights into the cellular biology of atherosclerosis. The Journal of cell biology 2015;209:13-22.

19. Pries AR, Badimon L, Bugiardini R, et al. Coronary vascular regulation, remodelling, and collateralization: mechanisms and clinical implications on behalf of the working group on coronary pathophysiology and microcirculation. European heart journal 2015;36:3134-46.

20. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation 1995;92:1355-74.

21. Libby P. The molecular mechanisms of the thrombotic complications of atherosclerosis. Journal of internal medicine 2008;263:517-27.

22. Takaya N, Yuan C, Chu B, et al. Association between carotid plaque characteristics and subsequent ischemic cerebrovascular events: a prospective assessment with MRI--initial results. Stroke 2006;37:818-23.

23. Fleg JL, Stone GW, Fayad ZA, et al. Detection of high-risk atherosclerotic plaque: report of the NHLBI Working Group on current status and future directions. JACC Cardiovasc Imaging 2012;5:941-55.

24. Nicoll R, Henein M. Arterial calcification: A new perspective? International journal of cardiology 2017;228:11-22.

25. Majesky MW. Developmental basis of vascular smooth muscle diversity. Arteriosclerosis, thrombosis, and vascular biology 2007;27:1248-58.

26. Liu R, Jin Y, Tang WH, et al. Ten-eleven translocation-2 (TET2) is a master regulator of smooth muscle cell plasticity. Circulation 2013;128:2047-57.

27. Owens GK. Regulation of differentiation of vascular smooth muscle cells. Physiological reviews 1995;75:487-517.

28. Shankman LS, Gomez D, Cherepanova OA, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis. Nat Med 2015;21:628-37.

29. Raines EW. PDGF and cardiovascular disease. Cytokine Growth Factor Rev 2004;15:237-54.

30. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. J Biol Chem 2009;284:3728-38.

31. Dubland JA, Francis GA. So Much Cholesterol: the unrecognized importance of smooth muscle cells in atherosclerotic foam cell formation. Curr Opin Lipidol 2016;27:155-61.

32. Feil S, Fehrenbacher B, Lukowski R, et al. Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. Circulation research 2014;115:662-7.

33. Tabas I, Glass CK. Anti-inflammatory therapy in chronic disease: challenges and opportunities. Science 2013;339:166-72.

34. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. Nat Rev Drug Discov 2012;11:860-72.

35. Bhak RH, Wininger M, Johnson GR, et al. Factors associated with small abdominal aortic aneurysm expansion rate. JAMA Surg 2015;150:44-50.

36. Shibamura H, Olson JM, van Vlijmen-Van Keulen C, et al. Genome scan for familial abdominal aortic aneurysm using sex and family history as covariates suggests genetic heterogeneity and identifies linkage to chromosome 19q13. Circulation 2004;109:2103-8.

37. Leeper NJ, Raiesdana A, Kojima Y, et al. Loss of CDKN2B promotes p53-dependent smooth muscle cell apoptosis and aneurysm formation. Arteriosclerosis, thrombosis, and vascular biology 2013;33:e1-e10.

38. Lederle FA, Johnson GR, Wilson SE, et al. Rupture rate of large abdominal aortic aneurysms in patients refusing or unfit for elective repair. JAMA 2002;287:2968-72.

39. Davis FM, Rateri DL, Daugherty A. Abdominal aortic aneurysm: novel mechanisms and therapies. Current opinion in cardiology 2015;30:566-73.

40. Back M, Gasser TC, Michel JB, Caligiuri G. Biomechanical factors in the biology of aortic wall and aortic valve diseases. Cardiovasc Res 2013;99:232-41.

41. Pearce WH, Zarins CK, Bacharach JM. Atherosclerotic Peripheral Vascular Disease Symposium II: controversies in abdominal aortic aneurysm repair. Circulation 2008;118:2860-3.

42. Bobadilla JL, Kent KC. Screening for abdominal aortic aneurysms. Adv Surg 2012;46:101-9.

43. Stather PW, Dattani N, Bown MJ, Earnshaw JJ, Lees TA. International variations in AAA screening. Eur J Vasc Endovasc Surg 2013;45:231-4.

44. Khosa F, Krinsky G, Macari M, Yucel EK, Berland LL. Managing incidental findings on abdominal and pelvic CT and MRI, Part 2: white paper of the ACR Incidental Findings Committee II on vascular findings. J Am Coll Radiol 2013;10:789-94.

45. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. Lancet 2008;371:1612-23.

46. Grau AJ, Weimar C, Buggle F, et al. Risk factors, outcome, and treatment in subtypes of ischemic stroke: the German stroke data bank. Stroke 2001;32:2559-66.

47. Bennett DA, Krishnamurthi RV, Barker-Collo S, et al. The global burden of ischemic stroke: findings of the GBD 2010 study. Glob Heart 2014;9:107-12.

48. Barnett HJ, Taylor DW, Eliasziw M, et al. Benefit of carotid endarterectomy in patients with symptomatic moderate or severe stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. N Engl J Med 1998;339:1415-25.

49. Halliday A, Harrison M, Hayter E, et al. 10-year stroke prevention after successful carotid endarterectomy for asymptomatic stenosis (ACST-1): a multicentre randomised trial. Lancet 2010;376:1074-84.

50. Chambers BR, Donnan GA. Carotid endarterectomy for asymptomatic carotid stenosis. Cochrane Database Syst Rev 2005:CD001923.

51. DeSantis CE, Lin CC, Mariotto AB, et al. Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin 2014;64:252-71.

52. Shahrokni A, Wu AJ, Carter J, Lichtman SM. Long-term Toxicity of Cancer Treatment in Older Patients. Clin Geriatr Med 2016;32:63-80.

53. Groarke JD, Nguyen PL, Nohria A, Ferrari R, Cheng S, Moslehi J. Cardiovascular complications of radiation therapy for thoracic malignancies: the role for non-invasive imaging for detection of cardiovascular disease. European heart journal 2014;35:612-23.

54. Jaworski C, Mariani JA, Wheeler G, Kaye DM. Cardiac complications of thoracic irradiation. Journal of the American College of Cardiology 2013;61:2319-28.

55. Darby SC, Ewertz M, McGale P, et al. Risk of ischemic heart disease in women after radiotherapy for breast cancer. N Engl J Med 2013;368:987-98.

56. Nilsson G, Holmberg L, Garmo H, et al. Distribution of coronary artery stenosis after radiation for breast cancer. J Clin Oncol 2012;30:380-6.

57. Gujral DM, Chahal N, Senior R, Harrington KJ, Nutting CM. Radiation-induced carotid artery atherosclerosis. Radiother Oncol 2014;110:31-8.

58. Abayomi OK. Neck irradiation, carotid injury and its consequences. Oral Oncol 2004;40:872-8.

59. Plummer C, Henderson RD, O'Sullivan JD, Read SJ. Ischemic stroke and transient ischemic attack after head and neck radiotherapy: a review. Stroke 2011;42:2410-8.

60. Halle M, Gabrielsen A, Paulsson-Berne G, et al. Sustained inflammation due to nuclear factor-kappa B activation in irradiated human arteries. Journal of the American College of Cardiology 2010;55:1227-36.

61. Halle M, Bodin I, Tornvall P, Wickman M, Farnebo F, Arnander C. Timing of radiotherapy in head and neck free flap reconstruction--a study of postoperative complications. Journal of plastic, reconstructive & aesthetic surgery : JPRAS 2009;62:889-95.

62. Halle M, Ekstrom M, Farnebo F, Tornvall P. Endothelial activation with prothrombotic response in irradiated microvascular recipient veins. Journal of plastic, reconstructive & aesthetic surgery : JPRAS 2010;63:1910-6.

63. Jacob F, Monod J. Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol 1961;3:318-56.

64. Mizuno T, Chou MY, Inouye M. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proceedings of the National Academy of Sciences of the United States of America 1984;81:1966-70.

65. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843-54.

66. Alexander RP, Fang G, Rozowsky J, Snyder M, Gerstein MB. Annotating non-coding regions of the genome. Nature reviews Genetics 2010;11:559-71.

67. Rosenbloom KR, Sloan CA, Malladi VS, et al. ENCODE data in the UCSC Genome Browser: year 5 update. Nucleic Acids Res 2013;41:D56-63.

68. Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. Cell 2014;157:77-94.

69. Beermann J, Piccoli MT, Viereck J, Thum T. Non-coding RNAs in Development and Disease: Background, Mechanisms, and Therapeutic Approaches. Physiological reviews 2016;96:1297-325.

70. Mattick JS, Rinn JL. Discovery and annotation of long noncoding RNAs. Nat Struct Mol Biol 2015;22:5-7.

71. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. Nature reviews Genetics 2016;17:47-62.

72. Liu G, Mattick JS, Taft RJ. A meta-analysis of the genomic and transcriptomic composition of complex life. Cell Cycle 2013;12:2061-72.

73. Djebali S, Davis CA, Merkel A, et al. Landscape of transcription in human cells. Nature 2012;489:101-8.

74. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nature reviews Genetics 2009;10:57-63.

75. Ambros V, Bartel B, Bartel DP, et al. A uniform system for microRNA annotation. RNA (New York, NY) 2003;9:277-9.

76. Pang KC, Frith MC, Mattick JS. Rapid evolution of noncoding RNAs: lack of conservation does not mean lack of function. Trends Genet 2006;22:1-5.

77. Hutvagner G, Zamore PD. RNAi: nature abhors a double-strand. Current opinion in genetics & development 2002;12:225-32.

78. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215-33.

79. Miller CL, Haas U, Diaz R, et al. Coronary heart disease-associated variation in TCF21 disrupts a miR-224 binding site and miRNA-mediated regulation. PLoS genetics 2014;10:e1004263.

80. Bastami M, Nariman-Saleh-Fam Z, Saadatian Z, et al. The miRNA targetome of coronary artery disease is perturbed by functional polymorphisms identified and prioritized by in-depth bioinformatics analyses exploiting genome-wide association studies. Gene 2016;594:74-81.

81. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic acids research 2014;42:D68-73.

82. Liu D, Chang X, Liu Z, Chen L, Wang R. Bistability and oscillations in gene regulation mediated by small noncoding RNAs. PloS one 2011;6:e17029.

83. Yanez-Mo M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles 2015;4:27066.

84. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circ Res 2010;107:1047-57.

85. Kaudewitz D, Zampetaki A, Mayr M. MicroRNA Biomarkers for Coronary Artery Disease? Curr Atheroscler Rep 2015;17:70.

86. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med 2014;20:460-9.

87. Zampetaki A, Willeit P, Tilling L, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. Journal of the American College of Cardiology 2012;60:290-9.

88. Maegdefessel L, Spin JM, Raaz U, et al. miR-24 limits aortic vascular inflammation and murine abdominal aneurysm development. Nature communications 2014;5:5214.

89. Janssen HL, Reesink HW, Lawitz EJ, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med 2013;368:1685-94.

90. Lorenzer C, Dirin M, Winkler AM, Baumann V, Winkler J. Going beyond the liver: progress and challenges of targeted delivery of siRNA therapeutics. J Control Release 2015;203:1-15.

91. Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. Nat Mater 2013;12:967-77.

92. Volpers C, Kochanek S. Adenoviral vectors for gene transfer and therapy. The journal of gene medicine 2004;6 Suppl 1:S164-71.

93. Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. Clinical microbiology reviews 2008;21:583-93.

94. Trono D. Lentiviral vectors: turning a deadly foe into a therapeutic agent. Gene therapy 2000;7:20-3.

95. Hacein-Bey-Abina S, Garrigue A, Wang GP, et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. The Journal of clinical investigation 2008;118:3132-42.

96. Foldvari M, Chen DW, Nafissi N, Calderon D, Narsineni L, Rafiee A. Non-viral gene therapy: Gains and challenges of non-invasive administration methods. Journal of controlled release : official journal of the Controlled Release Society 2016;240:165-90.

97. Boussif O, Lezoualc'h F, Zanta MA, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proceedings of the National Academy of Sciences of the United States of America 1995;92:7297-301.

98. Bragonzi A, Boletta A, Biffi A, et al. Comparison between cationic polymers and lipids in mediating systemic gene delivery to the lungs. Gene therapy 1999;6:1995-2004.

99. Song F, Yang D, Liu B, et al. Integrated microRNA network analyses identify a poor-prognosis subtype of gastric cancer characterized by the miR-200 family. Clinical cancer research : an official journal of the American Association for Cancer Research 2014;20:878-89.

100. Hunter AC. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. Advanced drug delivery reviews 2006;58:1523-31.

101. Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. The journal of gene medicine 2005;7:992-1009.

102. Wang D, Deuse T, Stubbendorff M, et al. Local MicroRNA Modulation Using a Novel Anti-miR-21-Eluting Stent Effectively Prevents Experimental In-Stent Restenosis. Arteriosclerosis, thrombosis, and vascular biology 2015.

103. Zhu X, Guo J, He C, et al. Ultrasound triggered image-guided drug delivery to inhibit vascular reconstruction via paclitaxel-loaded microbubbles. Sci Rep 2016;6:21683.

104. Park DH, Jung BK, Lee YS, et al. Evaluation of in vivo antitumor effects of ANT2 shRNA delivered using PEI and ultrasound with microbubbles. Gene therapy 2015;22:325-32.

105. Vodicka P, Smetana K, Jr., Dvorankova B, et al. The miniature pig as an animal model in biomedical research. Annals of the New York Academy of Sciences 2005;1049:161-71.

106. Davis BT, Wang XJ, Rohret JA, et al. Targeted disruption of LDLR causes hypercholesterolemia and atherosclerosis in Yucatan miniature pigs. PloS one 2014;9:e93457.

107. Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. Nature 2011;469:336-42.

108. Maegdefessel L, Azuma J, Toh R, et al. MicroRNA-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion. Sci Transl Med 2012;4:122ra22.

109. Cordes KR, Sheehy NT, White MP, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 2009;460:705-10.

110. Kriegel AJ, Liu Y, Fang Y, Ding X, Liang M. The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. Physiol Genomics 2012;44:237-44.

111. Maegdefessel L, Azuma J, Toh R, et al. Inhibition of microRNA-29b reduces murine abdominal aortic aneurysm development. The Journal of clinical investigation 2012;122:497-506.

112. Boon RA, Seeger T, Heydt S, et al. MicroRNA-29 in aortic dilation: implications for aneurysm formation. Circ Res 2011;109:1115-9.

113. Raaz U, Zollner AM, Schellinger IN, et al. Segmental aortic stiffening contributes to experimental abdominal aortic aneurysm development. Circulation 2015;131:1783-95.

114. Montgomery RL, Yu G, Latimer PA, et al. MicroRNA mimicry blocks pulmonary fibrosis. EMBO molecular medicine 2014;6:1347-56.

115. van Rooij E, Sutherland LB, Thatcher JE, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proceedings of the National Academy of Sciences of the United States of America 2008;105:13027-32.

116. Zhou Q, Chung AC, Huang XR, Dong Y, Yu X, Lan HY. Identification of novel long noncoding RNAs associated with TGF-beta/Smad3-mediated renal inflammation and fibrosis by RNA sequencing. The American journal of pathology 2014;184:409-17.

117. Alvarez ML, Khosroheidari M, Eddy E, Kiefer J. Role of microRNA 1207-5P and its host gene, the long non-coding RNA Pvt1, as mediators of extracellular matrix accumulation in the kidney: implications for diabetic nephropathy. PloS one 2013;8:e77468.

118. Leung A, Trac C, Jin W, et al. Novel long noncoding RNAs are regulated by angiotensin II in vascular smooth muscle cells. Circ Res 2013;113:266-78.

119. Bell RD, Long X, Lin M, et al. Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. Arteriosclerosis, thrombosis, and vascular biology 2014;34:1249-59.

120. Bartolomei MS, Zemel S, Tilghman SM. Parental imprinting of the mouse H19 gene. Nature 1991;351:153-5.

121. Dey BK, Pfeifer K, Dutta A. The H19 long noncoding RNA gives rise to microRNAs miR-675-3p and miR-675-5p to promote skeletal muscle differentiation and regeneration. Genes Dev 2014;28:491-501.

122. Deng Y, Yang Z, Terry T, et al. Prostacyclin-producing human mesenchymal cells target H19 lncRNA to augment endogenous progenitor function in hindlimb ischaemia. Nature communications 2016;7:11276.

123. Wang SH, Wu XC, Zhang MD, Weng MZ, Zhou D, Quan ZW. Upregulation of H19 indicates a poor prognosis in gallbladder carcinoma and promotes epithelial-mesenchymal transition. Am J Cancer Res 2016;6:15-26.

124. Raveh E, Matouk IJ, Gilon M, Hochberg A. The H19 Long non-coding RNA in cancer initiation, progression and metastasis - a proposed unifying theory. Mol Cancer 2015;14:184.

125. Kim DK, Zhang L, Dzau VJ, Pratt RE. H19, a developmentally regulated gene, is reexpressed in rat vascular smooth muscle cells after injury. The Journal of clinical investigation 1994;93:355-60.

126. Wang S, Aurora AB, Johnson BA, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Dev Cell 2008;15:261-71.

127. Zernecke A, Bidzhekov K, Noels H, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal 2009;2:ra81.

128. Jansen F, Yang X, Proebsting S, et al. MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease. Journal of the American Heart Association 2014;3:e001249.

129. Hartmann D, Fiedler J, Sonnenschein K, et al. MicroRNA-based Therapy of Gata2-deficient Vascular Disease. Circulation 2016.

130. Bonauer A, Carmona G, Iwasaki M, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science 2009;324:1710-3.

131. Boon RA, Hergenreider E, Dimmeler S. Atheroprotective mechanisms of shear stress-regulated microRNAs. Thromb Haemost 2012;108:616-20.

132. Hergenreider E, Heydt S, Treguer K, et al. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. Nat Cell Biol 2012;14:249-56.

133. Liao J, He Q, Li M, Chen Y, Liu Y, Wang J. LncRNA MIAT: Myocardial infarction associated and more. Gene 2016;578:158-61.

134. Yan B, Yao J, Liu JY, et al. lncRNA-MIAT regulates microvascular dysfunction by functioning as a competing endogenous RNA. Circulation research 2015;116:1143-56.

135. Tripathi V, Ellis JD, Shen Z, et al. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Molecular cell 2010;39:925-38.

136. Michalik KM, You X, Manavski Y, et al. Long noncoding RNA MALAT1 regulates endothelial cell function and vessel growth. Circulation research 2014;114:1389-97.

137. Lorenzen JM, Thum T. Long noncoding RNAs in kidney and cardiovascular diseases. Nature reviews Nephrology 2016;12:360-73.

138. Fiedler J, Breckwoldt K, Remmele CW, et al. Development of Long Noncoding RNA-Based Strategies to Modulate Tissue Vascularization. Journal of the American College of Cardiology 2015;66:2005-15.

139. Kanwal N, John P, Bhatti A. MicroRNA-155 as a therapeutic target for inflammatory diseases. Rheumatology international 2013;33:557-60.

140. Kurowska-Stolarska M, Alivernini S, Ballantine LE, et al. MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. Proceedings of the National Academy of Sciences of the United States of America 2011;108:11193-8.

141. Li X, Kong D, Chen H, et al. miR-155 acts as an anti-inflammatory factor in atherosclerosis-associated foam cell formation by repressing calcium-regulated heat stable protein 1. Scientific reports 2016;6:21789.

142. DuPont JJ, McCurley A, Davel AP, et al. Vascular mineralocorticoid receptor regulates microRNA-155 to promote vasoconstriction and rising blood pressure with aging. JCI insight 2016;1:e88942.

143. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proceedings of the National Academy of Sciences of the United States of America 2006;103:12481-6.

144. Hsu SD, Lin FM, Wu WY, et al. miRTarBase: a database curates experimentally validated microRNA-target interactions. Nucleic Acids Res 2011;39:D163-9.

145. Curtale G, Mirolo M, Renzi TA, Rossato M, Bazzoni F, Locati M. Negative regulation of Toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. Proceedings of the National Academy of Sciences of the United States of America 2013;110:11499-504.

146. Hulsmans M, Van Dooren E, Mathieu C, Holvoet P. Decrease of miR-146b-5p in monocytes during obesity is associated with loss of the anti-inflammatory but not insulin signaling action of adiponectin. PloS one 2012;7:e32794.

147. Raitoharju E, Lyytikainen LP, Levula M, et al. miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study. Atherosclerosis 2011;219:211-7.

148. Wang H, Jiang M, Xu Z, et al. miR-146b-5p promotes VSMC proliferation and migration. Int J Clin Exp Pathol 2015;8:12901-7.

149. Tsitsiou E, Lindsay MA. microRNAs and the immune response. Curr Opin Pharmacol 2009;9:514-20.

150. Schmidt WM, Spiel AO, Jilma B, Wolzt M, Muller M. In vivo profile of the human leukocyte microRNA response to endotoxemia. Biochem Biophys Res Commun 2009;380:437-41.

151. Roderburg C, Luedde M, Vargas Cardenas D, et al. Circulating microRNA-150 serum levels predict survival in patients with critical illness and sepsis. PloS one 2013;8:e54612.

152. Sun X, Sit A, Feinberg MW. Role of miR-181 family in regulating vascular inflammation and immunity. Trends in cardiovascular medicine 2014;24:105-12.

153. NE II, Heward JA, Roux B, et al. Long non-coding RNAs and enhancer RNAs regulate the lipopolysaccharide-induced inflammatory response in human monocytes. Nature communications 2014;5:3979.

154. Wilson JMG, Jungner G. Principles and Practice of Screening for Disease. Who Chronicle 1968;22:473-&.

155. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proceedings of the National Academy of Sciences of the United States of America 2008;105:10513-8.

156. Zubakov D, Boersma AW, Choi Y, van Kuijk PF, Wiemer EA, Kayser M. MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. Int J Legal Med 2010;124:217-26.

157. Jansen F, Yang X, Hoelscher M, et al. Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles. Circulation 2013;128:2026-38.

158. De Guire V, Robitaille R, Tetreault N, et al. Circulating miRNAs as sensitive and specific biomarkers for the diagnosis and monitoring of human diseases: promises and challenges. Clin Biochem 2013;46:846-60.

159. Jeck WR, Sorrentino JA, Wang K, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA (New York, NY) 2013;19:141-57.

160. Hansen TB, Jensen TI, Clausen BH, et al. Natural RNA circles function as efficient microRNA sponges. Nature 2013;495:384-8.

161. Simons PC, Algra A, van de Laak MF, Grobbee DE, van der Graaf Y. Second manifestations of ARTerial disease (SMART) study: rationale and design. Eur J Epidemiol 1999;15:773-81.

162. Schlosser FJ, Tangelder MJ, Verhagen HJ, et al. Growth predictors and prognosis of small abdominal aortic aneurysms. J Vasc Surg 2008;47:1127-33.

163. Perisic L, Aldi S, Sun Y, et al. Gene expression signatures, pathways and networks in carotid atherosclerosis. Journal of internal medicine 2015.

164. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol 2004;172:2731-8.

165. Yue F, Cheng Y, Breschi A, et al. A comparative encyclopedia of DNA elements in the mouse genome. Nature 2014;515:355-64.

166. McDonald RA, Halliday CA, Miller AM, et al. Reducing In-Stent Restenosis: Therapeutic Manipulation of miRNA in Vascular Remodeling and Inflammation. Journal of the American College of Cardiology 2015;65:2314-27.

167. Hinkel R, Penzkofer D, Zuhlke S, et al. Inhibition of microRNA-92a protects against ischemia/reperfusion injury in a large-animal model. Circulation 2013;128:1066-75.

168. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. The Journal of clinical investigation 2000;105:1605-12.

169. Manning MW, Cassis LA, Daugherty A. Differential effects of doxycycline, a broad-spectrum matrix metalloproteinase inhibitor, on angiotensin II-induced atherosclerosis and abdominal aortic aneurysms. Arteriosclerosis, thrombosis, and vascular biology 2003;23:483-8.

170. Daugherty A, Cassis LA. Mouse models of abdominal aortic aneurysms. Arteriosclerosis, thrombosis, and vascular biology 2004;24:429-34.

171. Anidjar S, Salzmann JL, Gentric D, Lagneau P, Camilleri JP, Michel JB. Elastase-induced experimental aneurysms in rats. Circulation 1990;82:973-81.

172. Pyo R, Lee JK, Shipley JM, et al. Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. The Journal of clinical investigation 2000;105:1641-9.

173. Azuma J, Asagami T, Dalman R, Tsao PS. Creation of murine experimental abdominal aortic aneurysms with elastase. J Vis Exp 2009.

174. Mestdagh P, Van Vlierberghe P, De Weer A, et al. A novel and universal method for microRNA RT-qPCR data normalization. Genome biology 2009;10:R64.

175. Lee CG, Da Silva CA, Dela Cruz CS, et al. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annual review of physiology 2011;73:479-501.

176. Mill C, George SJ. Wnt signalling in smooth muscle cells and its role in cardiovascular disorders. Cardiovasc Res 2012;95:233-40.

177. Lv L, Zhou Z, Huang X, et al. Inhibition of peptidyl-prolyl cis/trans isomerase Pin1 induces cell cycle arrest and apoptosis in vascular smooth muscle cells. Apoptosis 2010;15:41-54.

178. Ekstrand J, Razuvaev A, Folkersen L, Roy J, Hedin U. Tissue factor pathway inhibitor-2 is induced by fluid shear stress in vascular smooth muscle cells and affects cell proliferation and survival. J Vasc Surg 2010;52:167-75.

179. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med 2009;361:2449-60.

180. Stewart FA, Heeneman S, Te Poele J, et al. Ionizing radiation accelerates the development of atherosclerotic lesions in ApoE-/- mice and predisposes to an inflammatory plaque phenotype prone to hemorrhage. The American journal of pathology 2006;168:649-58.

181. Qin Q, Furong W, Baosheng L. Multiple functions of hypoxia-regulated miR-210 in cancer. J Exp Clin Cancer Res 2014;33:50.

182. Ludwig N, Leidinger P, Becker K, et al. Distribution of miRNA expression across human tissues. Nucleic acids research 2016;44:3865-77.

183. Swayze EE, Siwkowski AM, Wancewicz EV, et al. Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. Nucleic Acids Res 2007;35:687-700.

184. Xin M, Small EM, Sutherland LB, et al. MicroRNAs miR-143 and miR-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. Genes & development 2009;23:2166-78.

185. Waksman R, Pakala R. Drug-eluting balloon: the comeback kid? Circ Cardiovasc Interv 2009;2:352-8.

186. Kwekkeboom RF, Lei Z, Bogaards SJ, et al. Ultrasound and microbubble-induced local delivery of MicroRNA-based therapeutics. Ultrasound Med Biol 2015;41:163-76.